

# The role and regulation of prostaglandins in human tracheal epithelial cell lines

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# THE ROLE AND REGULATION OF PROSTAGLANDINS IN HUMAN TRACHEAL EPITHELIAL CELL LINES

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## Abstract

Evidence suggests induction and regulation of prostaglandin (PG) production is a key element in the pathophysiology of inflammatory disorders of the airways such as cystic fibrosis (CF). This study reports that human tracheal epithelial cell lines of a CF-phenotype, ΣCFTE29o$^-$, and a non CF-phenotype, 9HTEo$^-$, constitutively express the enzymes cyclooxygenase-1, cyclooxygenase-2, cytosolic prostaglandin E synthase and 15-hydroxyprostaglandin dehydrogenase at similar levels. Induction of these enzymes with pro-inflammatory cytokines could not be observed. $PGE_2$ production was significantly higher in ΣCFTE29o$^-$ cells than 9HTEo$^-$ cells.

Peroxisome proliferator-activated receptor γ (PPARγ) ligands are reported to down-regulate inflammatory mediator production in the airways. Additionally, 15-deoxy-$\Delta^{12,14}$- $PGJ_2$ (15d-$PGJ_2$) has been shown to induce expression of heme-oxygenase –1 (HO-1) in a number of cell systems. Tracheal epithelial cells were found to express PPARγ. PPARγ ligands did not affect expression of enzymes involved in $PGE_2$ generation in either ΣCFTE29o$^-$ or 9HTEo$^-$ cells. Troglitazone and 15d-$PGJ_2$ were observed to inhibit $PGE_2$ generation and transiently increase HO-1 expression by 9HTEo$^-$ cells. In ΣCFTE29o$^-$ cells the effects of PPARγ ligands on $PGE_2$ production were conflicting, increasing basal $PGE_2$ but inhibiting TNFα induction of this PG. Additionally, in ΣCFTE29o$^-$ cells, sustained induction of HO-1 expression could be observed in response to 15d-$PGJ_2$.

In summary, the findings of this study indicate that elevated levels of $PGE_2$ are a feature of the pro-inflammatory phenotype seen in CF. Furthermore, no significant difference in the expression or induction of enzymes involved in the biosynthesis of $PGE_2$ could be observed between the cell lines indicating that the source of elevated $PGE_2$ by the CF phenotype cell line is an elevated availability of substrate. The data included in this study also suggest that PPARγ ligands may be negative regulators of inflammation in non-CF airway epithelial cells, but in the CF airway epithelium may exacerbate existing inflammation.

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# Abbreviations

| | |
|---|---|
| 5-ASA | 5-Aminosalicyclic Acid |
| AA | Arachidonic Acid |
| Ab | Antibody |
| AcD | Actinomycin D |
| ANOVA | Analysis of Variance |
| AP | Activator Protein |
| APS | Ammonium Persulphate |
| ASL | Airway Surface Liquid |
| BADGE | Bisphenol diglycidyl ether |
| BAL | Bronchoalveolar Lavage |
| BALF | Bronchoalveolar Lavage Fluid |
| BSA | Bovine Serum Albumin |
| $Ca^{2+}$ | Calcium ions |
| C-C | Cysteine – Cysteine motif |
| cDNA | Complementary Deoxyribonucleic Acid |
| CF | Cystic Fibrosis |
| CFTR | Cystic Fibrosis Transmembrane Conductance Regulator |
| CHX | Cycloheximide |
| CLASS | Celecoxib Long-term Arthritis Safety Study |
| COX | Cyclooxygenase |
| CPGES | Cytosolic Prostaglandin E Synthase |
| $cPLA_2$ | Cytosolic Phospholipase $A_2$ |
| cpm | Counts per minute |
| CRE | cAMP Response Element |
| C-X-C | Cysteine – X – Cysteine motif |
| DAG | Diacylglycerol |
| ΔF508 | Omission of Phenylalanine residue at amino acid position 508 |
| DEPC | Diethyl Polycarbonate |
| DMEM | Dulbecco's Minimal Essential Medium |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EET | Epoxyeicosatetraenoic Acid |

| | |
|---|---|
| ELISA | Enzyme Linked Immunosorbent Assay |
| EnaC | Amiloride-sensitive epithelial Sodium Channel |
| ER | Endoplasmic Reticulum |
| ERK | Extracellular Signal Regulated Kinase |
| FBS | Foetal Bovine Serum |
| FCS | Foetal Calf Serum |
| GRO | Growth Related Gene Product |
| GTP | Guanosine Triphosphate |
| GTPase | Guanosine Triphosphatase |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid |
| HETE | Hydroxyeicosatetraenoic Acid |
| HPETE | Hydroperoxyeicosatetraenoic Acid |
| HPLC | High performance Liquid Chromatography |
| HO-1 | Heme-oxygenase-1 |
| Hsp | Heat Shock Protein |
| Hsp90 | Heat Shock Protein 90 |
| ICE | IL-1β Converting Enzyme |
| IFN | Interferon |
| IFNGR | Interferon Gamma Receptor |
| Ig | Immunoglobulin |
| IGF | Insulin Like Growth Factor |
| IκB | Inhibitor of κB |
| IKK | Inhibitor of κB Kinase complex |
| IL | Interleukin |
| IL-1R | Interleukin-1 Receptor |
| iNOS | Inducible Nitric Oxide Synthase |
| IRAK | IL-1 Receptor Associated Kinase |
| ISRE | Interferon Stimulated Response Element |
| IκB | Inhibitor of κB |
| JAK | Janus Kinase |
| kb | Kilo-Base |
| kDa | Kilo-Dalton |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharide |
| $LTB_4$ | Leukotriene $B_4$ |

| | |
|---|---|
| MAPK | Mitogen Activated Protein Kinase |
| MOPS | 3-[N-morpholino]propane-sulphonic acid |
| mPGES | Microsomal Prostaglandin E Synthase |
| mRNA | Messenger Ribonucleic Acid |
| MWt | Molecular Weight |
| NFκB | Nuclear Factor κ B |
| NIK | Nuclear Factor κ B Inducing Kinase |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NS-398 | N-(2-(Cyclohexyloxyl)-4-nitrophenyl) methanesulfonamide |
| NSAID | Non-Steroidal Anti-Inflammatory Drug |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PG | Prostaglandin |
| PGDH | 15-Hydroxy Prostaglandin Dehydrogenase |
| PGHS | Prostaglandin GH Synthase |
| $PGD_2$ | Prostaglandin $D_2$ |
| $PGE_2$ | Prostaglandin $E_2$ |
| $15d\text{-}PGJ_2$ | 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ |
| PH | Pleckstrin Homology |
| PKB | Protein Kinase B |
| PKC | Protein Kinase C |
| $PLA_2$ | Phospholipase $A_2$ |
| PLC | Phospholipase C |
| PMSF | Phenylmethylsulphonyl Fluoride |
| PPAR | Peroxisome-Proliferator Activated Receptor |
| PPRE | Peroxisome-Proliferator Response Element |
| RNA | Ribonucleic Acid |
| RXR | Retinoic Acid Receptor |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis |
| SEM | Standard Error of the Mean |
| $sPLA_2$ | Secretory Phospholipase $A_2$ |
| STAT | Signal Transducer and Activator of Transcription |
| TACE | Tumour Necrosis α Converting Enzyme |

| | |
|---|---|
| TATA | Adenine Thymine rich promoter sequence |
| TBS | Tris Buffered Saline |
| TEMED | N',N',N',N'-tetramethylethylene diamine |
| TGF | Transforming Growth Factor |
| TNF | Tumour Necrosis Factor |
| TNFR1 | Tumour Necrosis α Receptor 1 |
| TNFR2 | Tumour Necrosis α Receptor 2 |
| TRAF6 | Tumour Necrosis Receptor-Associated Factor 6 |
| Tween-20 | Polyoxyethylenesorbitan monolaurate |
| $TXA_2$ | Thromboxane A2 |
| UTR | Untranslated Region |
| UV | Ultraviolet |
| VIGOR | Vioxx Gastrointestinal Outcomes Research |

# Single letter amino acid codes

| | |
|---|---|
| A | Ala |
| C | Cys |
| D | Asp |
| E | Glu |
| F | Phe |
| G | Gly |
| H | His |
| I | Ile |
| K | Lys |
| L | Leu |
| M | Met |
| N | Asn |
| P | Pro |
| Q | Gln |
| R | Arg |
| S | Ser |
| T | Thr |
| V | Val |
| W | Trp |
| Y | Tyr |

# 1. Introduction

## 1.1 Airway Inflammation

### 1.1.1 Inflammation and the Airway Epithelium

The acute-inflammatory response is a key defense mechanism by which an organism protects itself from invading pathogens and irritants. Inflammation is a complex process initiated by tissue damage and is, by large, protective. The inflammatory response consists of two components: an innate response with no immunological basis, consisting of vascular and cellular events orchestrated by chemical mediators released from cells and plasma; and a complex acquired response involving lymphocytes that recognises self from non-self and is specific for the invading pathogen (Reviewed in Bevins, 1999). Ordinarily, the outcome of an inflammatory reaction is eradication of the pathogen and healing of the host tissue. However, if inappropriately deployed or if the causative agent persists the inflammatory response can be deleterious resulting in damage to the host. Chronic inflammation is a feature of a number of diseases of the airways such as cystic fibrosis, asthma, and chronic obstructive pulmonary disease.

The airway epithelium represents the largest epithelial surface of the body in contact with the external environment and functions to minimise microbial adherence and provide a hostile milieu for potential pathogens. The airway epithelium is a physical barrier that employs a number of host defence mechanisms to prevent infection and stimulation of sensory nerves and smooth muscle by inhaled irritants. Airway epithelial cells participate in an inflammatory response in a number of ways; They can act as target cells, modulating one or several of their functions, such as mucin secretion, ciliary beating and ion transport. Additionally, they can act as effector cells, synthesising a number of agents such as cytokines, lipid mediators and reactive oxygen species affecting both the epithelium and neighbouring tissues and contributing to or inhibiting airway inflammation (Reviewed by Adler *et al.,* 1994). Aberrations in any of these functions, as seen with environmental exposure or a number of disease states such as cystic fibrosis and asthma can result in an impaired pulmonary response and damage to the host tissue. A number of organ and cell culture systems have been implemented

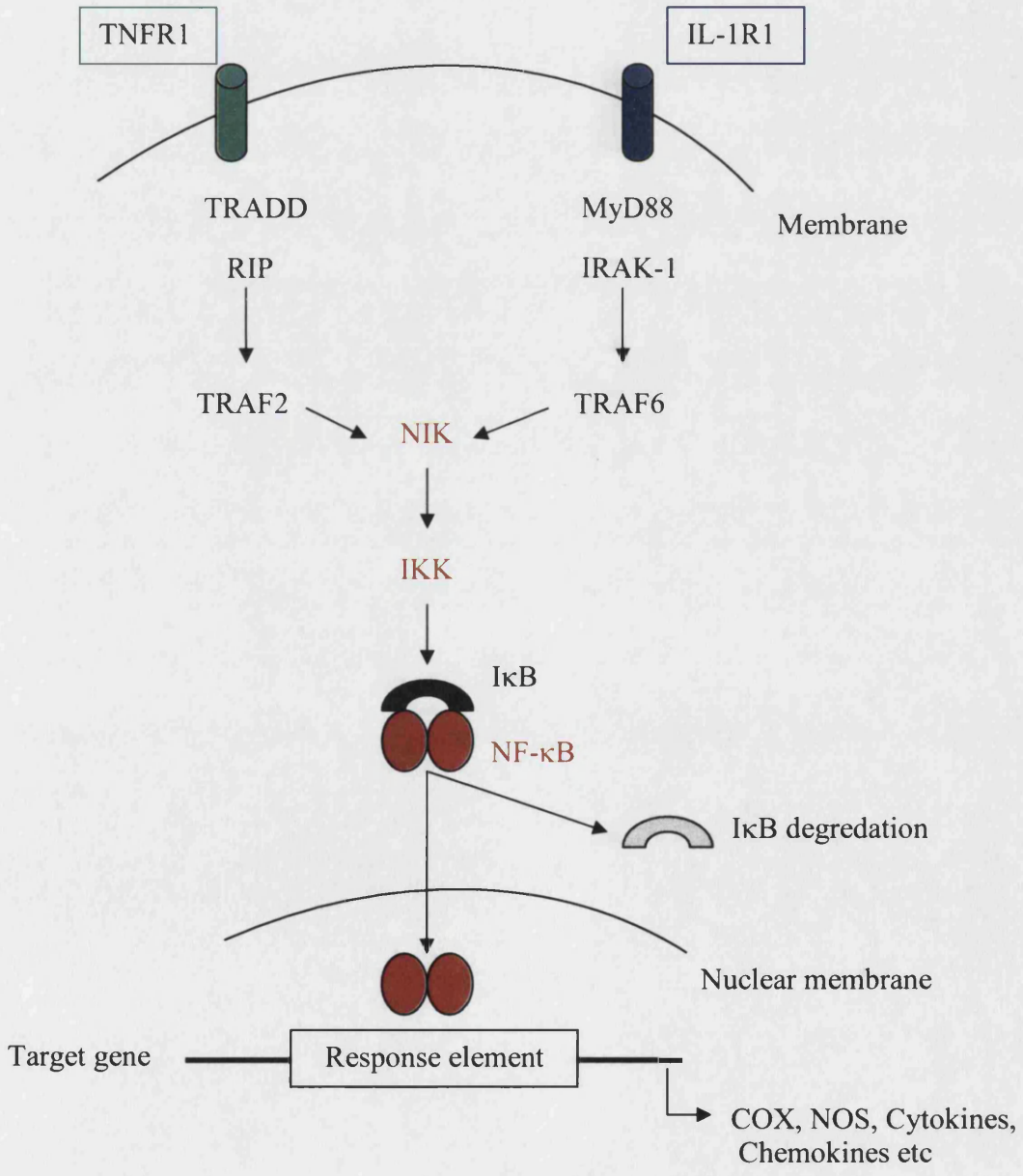
in order to study the response of the airway epithelium to infection. These studies have revealed two complementary defense mechanisms involving first the production of anti-microbial agents and secondly the induction of signalling networks that recruit phagocytic cells to the site of infection (Diamond *et al.,* 2000).

Damaged tissues release mediators including vasoactive peptides, eicosanoids, chemokines, cytokines and nitric oxide (NO) resulting in activation of surrounding cells, induction of vasodilatation and exudation of fluid. The fluid exudate is carried to the lymph glands where products of the invading microorganism trigger an immune response. Additionally, some of these mediators are chemoattractants for blood derived cells such as neutrophils, eosinophils, monocytes and lymphocytes (Zhang *et al.,* 2000). Vasoactive peptides such as bradykinin (BK) are formed, during the inflammatory response, from α2-globulins and high and low molecular weight kininogens by the action of kininogenases (Reviewed by Barnes *et al.,* 1998). In the airways BK acts at B2 receptors triggering release of $PGE_2$ and NO from the airway epithelium (Ricciardolo *et al.,* 1996) and is capable of causing both constriction or relaxation of airway smooth muscle. BK is also a potent vasodilator of bronchial vessels and induces microvascular leakage. Additionally, BK stimulates mucus secretion from human sub-mucosal glands and is a potent mediator of pain, manifested in the airways as cough and tightness of the chest (Fuller *et al.,* 1987). A wide range of anti-microbial substances including lysozyme, lactoferrin, phospholipase $A_2$, defensins, proteases and complement can be found in the mucus layer covering airway epithelia (Bals *et al.,* 1999). However, the epithelial fluid layer is very thin under resting conditions, and the commonly used bronchoalveolar lavage (BAL) method combines secretions from anatomically distinct locations, making the assessment of the contribution of individual proteins or peptides to the innate immune response challenging (Reviewed in Ganz, 2002). Nonetheless, these components are postulated to act in synergy to kill bacteria and prevent infection. Furthermore, mediators of the inflammatory response such as cytokines, chemokines, COX products and NOS products can further stimulate airway epithelial cells inducing mucus hypersecretion enabling effective eradication of pathogens (Borchers *et al.,* 1999). The role of eicosanoids in inflammation, and in particular airway inflammation, will be discussed in detail in the next section. Chemokines are important chemotactic cytokines that play a

fundamental role in the trafficking of leukocytes to sites of inflammation. They are also potent cell-activating factors, inducing cytokine and histamine release and free radical production, a fact that makes them particularly important in the pathogenesis of allergic inflammation. Much of the inflammatory response is orchestrated by cytokines. Cytokines are soluble extracellular signalling proteins that form a network of cell-cell communication regulating growth, differentiation and survival of cells; the initiation, maintenance and resolution of the innate immune response and subsequent activation of the adaptive immune system (Strieter *et al.,* 2001). Cytokines affect cellular activity by binding to cell-surface receptors, on immune or non-immune target cells, and a signal being transduced to the nucleus inducing transcription of genes. Airway epithelial cells have been observed to produce, express receptors for, and be responsive to a number of proinflammatory cytokines including interleukin-1 (IL-1) and tumour necrosis factor (TNF), and interferon gamma (IFNγ) (Mitchell *et al.,* 1994; Asano *et al.,* 1996; Watkins *et al.,* 1999; Rodgers *et al.,* 2002; Reibman *et al.,* 2003). The IL-1 family consists of two agonists IL-1α and IL-1β that bind to the IL-1 type one receptor (IL-1R1). Upon binding of IL-1 to IL-1R1 a receptor-associated protein, MyD88, recruits an adapter protein, which in turns recruits the IL-1 associated kinase (IRAK). Subsequent to activation, IRAK activates TNF receptor-associated factor 6 (TRAF6) which complexes with nuclear factor- κB-inducing kinase (NIK). NIK activates the IκB kinase complex (IKK) which phosphorylates inhibitor of κBα (IκBα) resulting in ubiquitination and degradation of inhibitor κB (IκB). The transcription factor, nuclear factor- κB (NF-κB) is then free to translocate to the nucleus and transactivate numerous target genes by binding to responsive elements in the DNA (Figure 1). NF-κB induces the transcription of genes encoding many proteins involved in the inflammatory response, including cyclooxygenase, chemokines, cytokines and NO-synthase (Murphy *et al.,* 2000). IL-1 can also signal through the p38 mitogen-activated protein (MAP) and the c-Jun N-terminal kinase (JNK) pathways, their effects synergising with those of NF-κB. TNF signals through two distinct receptors TNRF1 and TNRF2. As with IL-1, TNF signalling is mediated by NIK and MAP activation of NF-κB (Figure 1) (Reviewed in Strieter *et al.,* 2001). The production of these cytokines by the airway epithelium not only induces the production of mediators of the host defense such as COX, NOS and cytokines but can indirectly regulate inflammatory cell recruitment. TNF and IL-1 do not directly induce migration of neutrophils, but both cytokines are potent inducers for

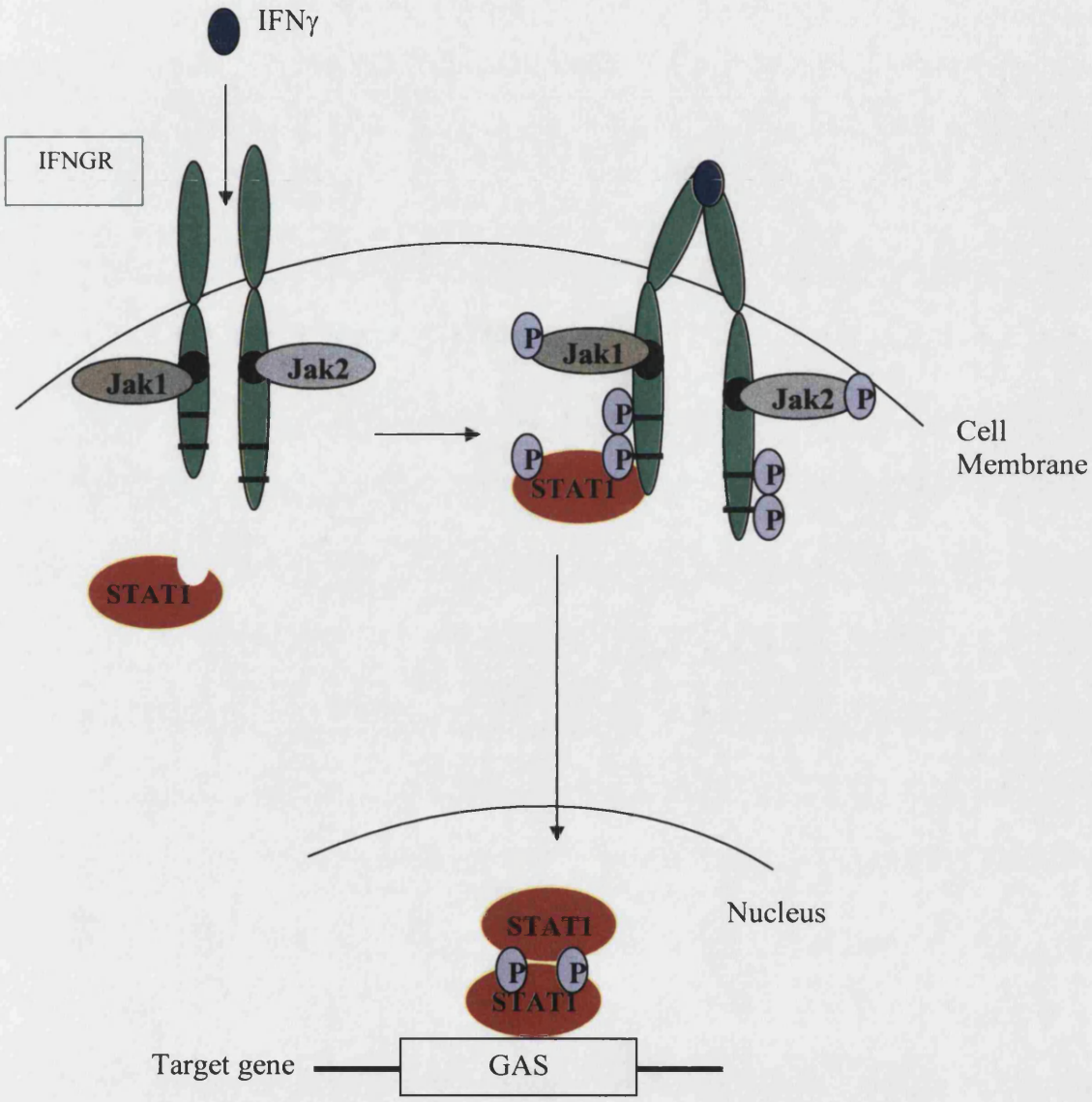
the production of C-X-C chemokines, such as IL-8, by the airway epithelium, macrophages and other cells of the airways. The C-X-C chemokines are the major chemoattractants for inflammatory cells (Kunkel *et al.*, 1991).

**Figure 1** IL-1 and TNF signal transduction: Activation and translocation of NF-κB resulting in transactivation of a number of genes involved in the inflammatory response. TRADD, TNFR1-associated death domain; RIP, Receptor-interacting protein.



IFNγ is a proinflammatory cytokine predominantly derived from lymphoid cells (Reviewed in Frucht *et al.,* 2001). Bader and Nettesheim report that the tracheal epithelium can be stimulated to produce low levels of IFNγ 4 hours after exposure to TNFα and that, in response to TNFα, the airway epithelium releases a pattern of inflammatory mediators that resembles a Th1 response (Bader and Nettesheim, 1996). IFNγ exerts its activity by binding to and activating the IFNγ receptor (IFNGR), which comprises of two transmembrane domains (Aguet, 1988). Upon binding of IFNγ to the IFNGR the sub-units of the receptor dimerize bringing associated Janus kinases (Jaks), Jak1 and Jak2, into close proximity resulting in their tyrosine phosphorylation and increasing their catalytic activity. The phosphorylation of the Jaks correlates with tyrosine phosphorylation of the IFNGR components and recruitment of the transcription factor signal transducer and activator of transcription 1 (STAT1). STAT1 is phosphorylated at the conserved carboxy-terminal tyrosine residue (Reviewed in Ramana *et al.,* 2002) and subsequently, homo- or heterodimers of STAT translocate to the nucleus and interact with a gamma activation site (GAS) modifying transcription and expression of target genes (Figure 2) (Schindler and Darnell, 1995). IFNγ plays a pivotal role in the host inflammatory response potentiating the respiratory burst and activity of professional phagocytes and enhancing generation of inflammatory mediators such as nitric oxide (NO) (Barnes and Liew, 1995). Additionally, a number of studies have reported that cytokines IL-1, TNF and IFNγ can act in concert to produce an effect. Martin *et al* report that, while individually IL-1, TNF and IFNγ fail to have an effect upon thin lung slices, in combination these cytokines induce contraction of the airways by inducing up-regulation of COX-2 expression and thromboxane release. This study also reports that challenge with cytokines can produce either dilatation or constriction of airway smooth muscle depending upon the time point after introduction of the cytokine, demonstrating the ability of the airway epithelium to participate in an integrated inflammatory response and the importance of the time dependent effects of inflammatory mediators released by the airway epithelium (Martin *et al.,* 2001).

**Figure 2** IFNγ signal transduction: Activation and translocation of STAT1 resulting in transactivation of a number of genes involved in the inflammatory response.




The ability of the airway epithelium not only to respond to inflammatory mediators by altering one or several of its functions, but to generate many substances that may diffuse away and affect neighbouring cells and tissues, underlines its critical importance as not only a primary defense against pathogens and infection, but as an orchestrator of the hosts innate defense able to translate gene-environment interactions.

### 1.1.2 Cystic Fibrosis

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder affecting Caucasian populations. In Northern Europe the disease frequency is approximately 1:2500 live births (Boat *et al.,* 1989; Collins, 1992). CF was first identified as a distinct disease in 1938 (Anderson, 1938). However, literature recording infant cases of meconium ileus and pancreatic and lung disease typical of CF date as far back as 1650 (Boat *et al.,* 1989). In 1938 few patients lived past the age of five years and most died of rapidly progressive pulmonary infections (Colten, 1995). In the following decades the use of antibiotic treatments and early detection of the disease led to a steadily increasing number of CF patients reaching adulthood. In 1983 Paul Quinton showed that chloride uptake by absorptive sweat duct epithelium was abolished in CF, this characteristic is now used as a diagnostic tool for the disease. Improved nutrition, the ability to detect CF at early and presymptomatic stages along with effective antibiotic therapy have improved both the quality and duration of the CF patient's life. In 2001 the median life expectancy of patients born in 1990 was 40 years of age (Jaffe, 2001).

CF affects several organ systems including the pancreas, gastro-intestinal tract and the reproductive system. However, chronic infection and deterioration of lung function are the major causes of morbidity and death. An imbalance in the ionic and/or osmotic balance of the airway surface liquid (ASL) results in clogging of the airways with thick, sticky mucus and facilitates subsequent colonisation by bacteria such as *Pseudomonas aeruginosa,* a bacteria associated with progressive pulmonary deterioration and untimely death (Bals *et al.,* 1999).

Studies in the 1980s identified defects in the chloride conductance properties of epithelial cells from CF patients (Quinton, 1983) and subsequently that this defect was localised to the apical membrane (Widdicombe *et al.,* 1985). In 1989 the CF gene was identified and cloned (Riordan *et al.,* 1989; Rommens *et al.,* 1989). Analysis of the predicted gene product revealed a 1480 amino acid glycosylated transmembrane glycoprotein that functions as a cyclicAMP-regulated chloride channel (Bals *et al.,* 1999). The gene product was called the CF transmembrane conductance regulator (CFTR) because of the link between abnormal chloride ion transport and the CF defect. The CFTR consists of two homologous halves, each half comprising of six

transmembrane domains and a nucleotide-binding domain (NBD). The two halves of the CFTR are linked via a regulatory (R) domain that contains a number of consensus phosphorylation sites (Riordan *et al.,* 1989). The CFTR is predominantly found in epithelial cells where it is transcribed at low concentrations, and found at highest levels in the pancreas, proximal lung tissue, salivary glands, sweat ducts, intestine and reproductive tract (Collins, 1992).

Gating of the CFTR channel is controlled by two separate processes 1) phosphorylation and 2) binding and hydrolysis of ATP. Serine residues in the R domain are phosphorylated by cAMP-dependent protein kinase (PKA) the greater the phosphorylation, the greater the probability the channel will open (Akabas, 2000). Following phosphorylation of the R domain ATP binds to and is hydrolysed in the NBDs (Winter and Welsh, 1997). Channel opening allows conduction of anions, particularly $Cl^-$ ions, across the apical membrane of epithelial cells. The channel is subsequently deactivated by the action of phosphatases PP2C and PP2A (Vankeerbergen *et al.*, 2001). To date, over 1,000 mutations of the CFTR gene have been associated with a CF disease phenotype (Reviewed by Wagner and Headley, 2003). Mutations of the CFTR can be grouped into five classes (I-IV) on the basis of CFTR alterations (Reviewed in Zeitlin, 1999) (Table 1).

The CFTR regulates the activities of amiloride sensitive $Na^+$ channels, outwardly rectifying $Cl^-$ channels; the $Cl^-/HCO_3$ exchanger and the potassium channel Kir 1.1 (ROMK) (Akabas, 2000). Hypotheses on the mechanisms by which mutated CFTR affects the function of lung epithelia consider the role of the CFTR in salt absorption to be of cardinal importance. The high salt hypothesis (Smith, 1996, Zabner, 1998) postulates that reduced transepithelial $Cl^-$ conductance results in high salt levels in the ASL that interferes with "salt-sensitive" natural antibiotics and lysozyme. The low volume hypothesis (Matsui *et al.,* 1998) suggests that both CF and normal airway ASL contain comparable levels of salt, however, in CF airways the volume of liquid is reduced because of an increased isotonic fluid absorption resulting in thick mucus leading to obstruction and infection. Recent *in vivo* evidence supports the "low volume hypothesis" and provides evidence that, in CF, ASL is approximately isotonic and does not contain a high level of salt (Jayaraman, 2001).

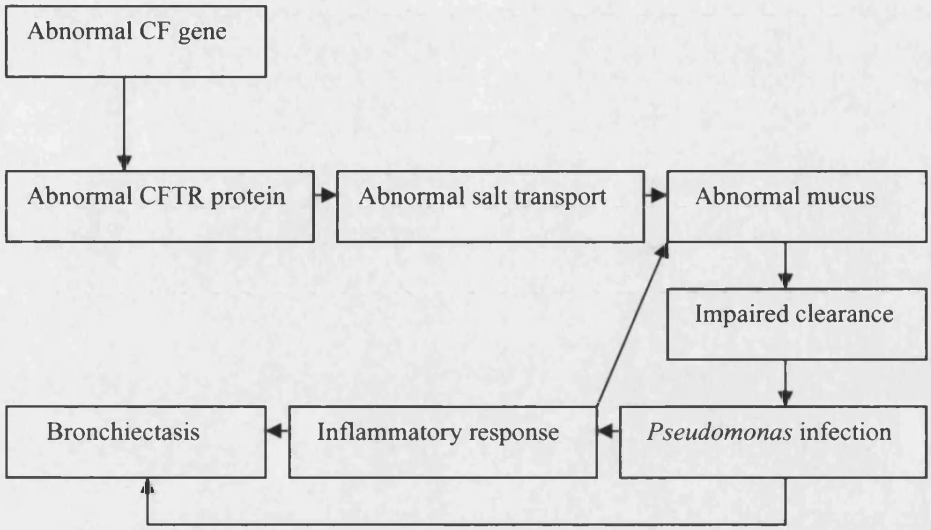
**Table 1** Classification of mutations that may lead to CF

| Group | Mutation |
|---|---|
| **Class I** | Synthesis of unstable or truncated CFTR RNA, resulting in total absence of CFTR protein/function. **Severe disease phenotype**. |
| **Class II** | Protein translation is completed, but an abnormal protein that fails to escape the ER is produced. Responsible for approximately 70% of patients. **Severe disease phenotype.** |
| **Class III** | Disrupt activation and regulation of CFTR at the plasma membrane. The channel is postulated to be defective with respect to ATP binding and hydrolysis, or phosphorylation. **Severe disease phenotype**. |
| **Class IV** | Chloride conductance or channel gating are affected resulting in a reduced chloride current. **Mild disease phenotype.** |
| **Class V** | Decrease in the level of normal CFTR due to alterations in the promoter or alterations in splicing. **Reduction in mRNA to <10% results in a disease phenotype.** |

### 1.1.3 Cystic Fibrosis and Airway inflammation

The generation of an inflammatory response is a crucial mechanism by which the individual protects itself from infectious and non- infectious insults. During the last two decades a number of studies have identified the host inflammatory response as a potential target to delay the deterioration of lung function that occurs in patients with CF. An intrinsic component of the CF phenotype is a persistent and exaggerated inflammatory response driven by bacterial infection of the airways (Figure 3).

**Figure 3** The basis of respiratory complications in CF (Adapted from Collins, 1992)




Epithelial cells respond to bacteria and bacterial products by increasing production of cytokines such as TNF, IL1 and IFNγ and IL-8. IL-8 is a potent chemokine that attracts neutrophils to the inflammatory site. Furthermore, when macrophages present encounter bacteria they respond by producing their own IL-8, TNF-α and IL-1β which, in turn, further drives the epithelial cells to produce molecules that participate in the inflammatory response (Reviewed by Chmiel and Davis, 2003). The airways of CF patients are often colonised early in life by a limited spectrum of bacteria and, in response, mount a vigorous inflammatory response. Initially this response contains the bacteria and the infection is cleared. Intermittent colonisation of the airways of CF patients is common in the first few years of life and in a clinical trial Dakin *et al.* demonstrate, that in infants and young children with CF, the presence of pathogen in the lower airways correlates with levels of inflammation (Dakin *et al.,* 2002). However, eventually the CF lung is unable to clear infection, and bacterial colonisation first by *Staphylococcus aureus* and then *Pseudomonas aeruginosa* becomes chronic. Konstan *et al.* report that, in older children and adults with CF, even clinically stable patients have large numbers of bacteria in their lower airways accompanied by a neutrophil influx and uninhibited elastase activity (Konstan *et al.,* 1994).

Evidence suggests that the inflammatory process goes awry in the lungs of CF patients even in infancy and is ultimately ineffective against the bacteria and damaging to the host tissue. Clinical studies indicate that the neutrophil and IL-8 responses measured in bronchoalveolar lavage (BAL) fluid of CF infants are excessive when compared to normal infants (Muhlebach *et al.,* 1999). While the subjects observed in the aforementioned study appear to be responding to bacteria, other studies of inflammatory mediators in BAL fluid have revealed that many CF infants have no detectable bacteria, but nonetheless have a modest inflammatory response that exceeds that observed in uninfected non-CF subjects (Khan *et al.,* 1995). These findings suggest that, in response to bacterial stimulation, the CF lung mounts an excessive inflammatory response that is not down-regulated once the infection has been eliminated (Reviewed by Chmiel and Davis, 2003). The excessive inflammatory response seen in CF has also been observed in studies using mice. In 1997 Van Heeckeren *et al.* published findings that CF mice with chronic *Pseudomonas* infection have a higher mortality than their non-CF littermates. Furthermore, the BAL of the CF mice contained excess pro-inflammatory cytokines, in response to the same bacterial burden, when compared to the non-CF mice (Van Heeckeren *et al.,* 1997). This is supported by the findings of Thomas *et al.,* who investigating S100-A8, a calcium binding protein identified as a potent pro-inflammatory chemotactic factor, observed that CF-mice show a 4-fold higher level of S100-A8 mRNA accompanied by an increased influx of neutrophils and increased production of TNF-α in response to bacterial lipopolysaccharide (LPS) than their non-CF littermates. These results indicate that the pathology of CF may relate to an underlying hypersensitivity of the immune system to bacterial challenge (Thomas *et al.,* 2000). However, other clinical studies show that at least some CF infants, particularly those that have no known history of lung infection, do not exhibit an elevated / detectable inflammatory response and that in young children airway inflammation improves when the pathogen is eradicated (Armstrong *et al.,* 1997).

A persistent and exaggerated inflammatory response, associated with an abnormal increase in pro-inflammatory cytokines, is one of the major factors leading to damage of the host tissue in CF (Escotte *et al.,* 2002). Tiddens *et al.* report that in CF patients undergoing lung transplantation up to 23% of the airway was not covered by epithelium (Tiddens *et al.*, 2000). When the airway epithelium is breached the airways are

rendered susceptible to injury by inhaled pathogens and particles (Reviewed in Whitsett, 2002). It has been shown that *Pseudomonas aeruginosa* preferentially adheres to regenerating airway epithelium (de Bentzmann *et al.,* 1996). Furthermore, Muraoka *et al.* demonstrate that increased activity of the transcription factor NF-κB inhibits growth of the airway epithelium (Muraoka *et al.,* 2000), these findings are of interest when taken into consideration with the findings of Venkatakrishnan *et al.* who report that activation of NF-κB is exaggerated in CF bronchial epithelial cells (Venkatakrishnan *et al.,* 2000). These studies suggest that NF-κB may inhibit epithelial repair and provide evidence of a role for NF-κB in the exaggerated inflammatory response seen in CF airways.

Injury to the airway epithelium combined with viscous mucus and the failure of airway epithelial cells to ingest and inactivate bacteria contribute to the ability of bacteria to colonise the airways of CF patients. Chronic infection of the airways with pathogens such as *Pseudomonas aeruginosa* stimulates a vigorous, but ultimately ineffective, inflammatory response that damages the host tissue and results in permanent ongoing lung destruction (Reviewed in Dinwiddie, 2000).

## 1.2 Prostaglandins

### 1.2.1 History

Interest in the prostagandins dates back to the early 1930s when Kurzok and Lieb observed that strips of uterine smooth muscle could be contracted by the exogenous application of semen (Kurzok and Lieb, 1930). The substance in semen that induced contraction of the uterine smooth muscle was believed to originate in the prostate and subsequently given the misnomer 'prostaglandin' by von Euler in 1936. More than two decades later it became apparent that prostaglandin was in fact a family of 20-carbon unsaturated carboxylic acids with a cyclopentenone ring, and their kinship with essential fatty acids recognised.

It is now known that prostaglandins are produced by almost every tissue in the human body, their production being increased or decreased in response to a diverse range of stimuli. The effects of the prostaglandins influence practically every biological function and since the discovery that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, exert their activity by inhibiting prostaglandin production (Vane, 1971) there has been intense interest in their metabolic pathway and its role in both physiology and pathophysiology.

### 1.2.2 The Cyclooxygenases

Cyclooxygenase (COX), also known as prostaglandin H synthase, is widely accepted to exist in two isoforms COX-1 and COX-2, the products of two distinct genes localised on chromosomes 9 and 1 respectively (Funk, 1991 and Tay, 1994). COX-1 was purified from ovine and bovine seminal vesicles (Hemler *et al.*, 1976; Miyamato *et al.*, 1976; van de Ouderaa *et al.*, 1977) and is generally thought to be a constitutive isoform acting to maintain homeostatic processes such as mucus secretion, platelet aggregration and parturition (Reviewed by Smith and Langenbach, 2001). COX-2 was discovered more recently (Xie *et al.*, 1991; Kujubu *et al.*, 1991; O'Banion *et al.*, 1991) and is mainly an inducible enzyme involved in cell proliferation and inflammatory processes,

but also has a role in physiological processes such as ovulation and implantation and neonatal development (Reviewed by Smith and Langenbach, 2001). Sequence analysis of the COX-2 5'-flanking region has revealed potential transcription regulatory elements including a TATA box, two NF-κB sites, two AP-2 sites and a CRE motif (Tanbe and Tohnai, 2002). Additionally, COX-2 mRNA has multiple AUUUA instability sequences dictating rapid degradation and short half-life (Ristimaki *et al.,* 1994). Recently evidence has been published supporting the presence of a third distinct acetominophen sensitive COX isoform, COX-3, a splice variant of COX-1 that shares all of the important catalytic and structural features of COX-1 and COX-2 (Simmons *et al.,* 1999; Chandrasekharan *et al.,* 2002).

COX is the rate-limiting enzyme for the conversion of arachidonic acid (AA) to prostanoids. AA is a 20-carbon unsaturated fatty acid containing four double bonds and is either derived from dietary linoleic acid or ingested as a component of meat, it is then esterified as a component of the phospholipids of cell membranes to be released into the cytosol by the action of the enzyme phospholipase $A_2$ ($PLA_2$). Liberated AA is converted into prostaglandin (PG) $G_2$ and then $PGH_2$ by the COXs. COX enzymes are bifunctional, catalysing two sequential reactions in spatially distinct, but mechanistically linked active sites (Kiefer *et al.,* 2000). Initially COX catalyses the insertion of molecular oxygen into AA to form the unstable intermediate $PGG_2$, which is rapidly converted into $PGH_2$ by the peroxidase activity of COX. $PGH_2$ is subsequently converted into biologically active PGs by their relevant synthases (Figure 5) (Reviewed by Subbaramaiah and Dannenberg, 2002). As previously mentioned the PGs participate in a diverse range of physiological and pathophysiological processes. It is the role of the PGs in pathophysiological processes, in particular inflammation, that makes the COX enzymes important therapeutic targets.

In 1893, Felix Hoffman, at the Bayer company, set into motion the commercial process to produce acetylsalicylic acid (aspirin) a palatable form the active component of willow bark, salicylic acid, which has been used by man for thousands of years to relieve pain and fever. This lead to the development of the class of drugs termed the NSAIDs, which are still the most widely used therapeutic agents over a century later (Reviewed by Dubois *et al.*, 1998). In 1971 Vane showed that the anti-inflammatory action of NSAIDs, such as aspirin, rests in their ability to inhibit the activity of the COX

enzyme, resulting a dose-dependent reduction in PG production (Vane, 1971). NSAIDs act at the COX active site in several ways; Aspirin irreversibly inactivates both COX-1 and COX-2 by acetylating an active-site serine, this covalent modification interferes with the binding of AA at the COX active site. NSAIDs including ibuprofen reversibly inhibit COX activity by competing with AA for the COX active site and thirdly a group of NSAIDs that includes indomethacin causes a time-dependent reversible inhibition of COX resulting from the formation of a salt bridge between the carboxylate of the drug and arginine 120 followed by conformational changes (Reviewed by Hinz and Brune, 2002).

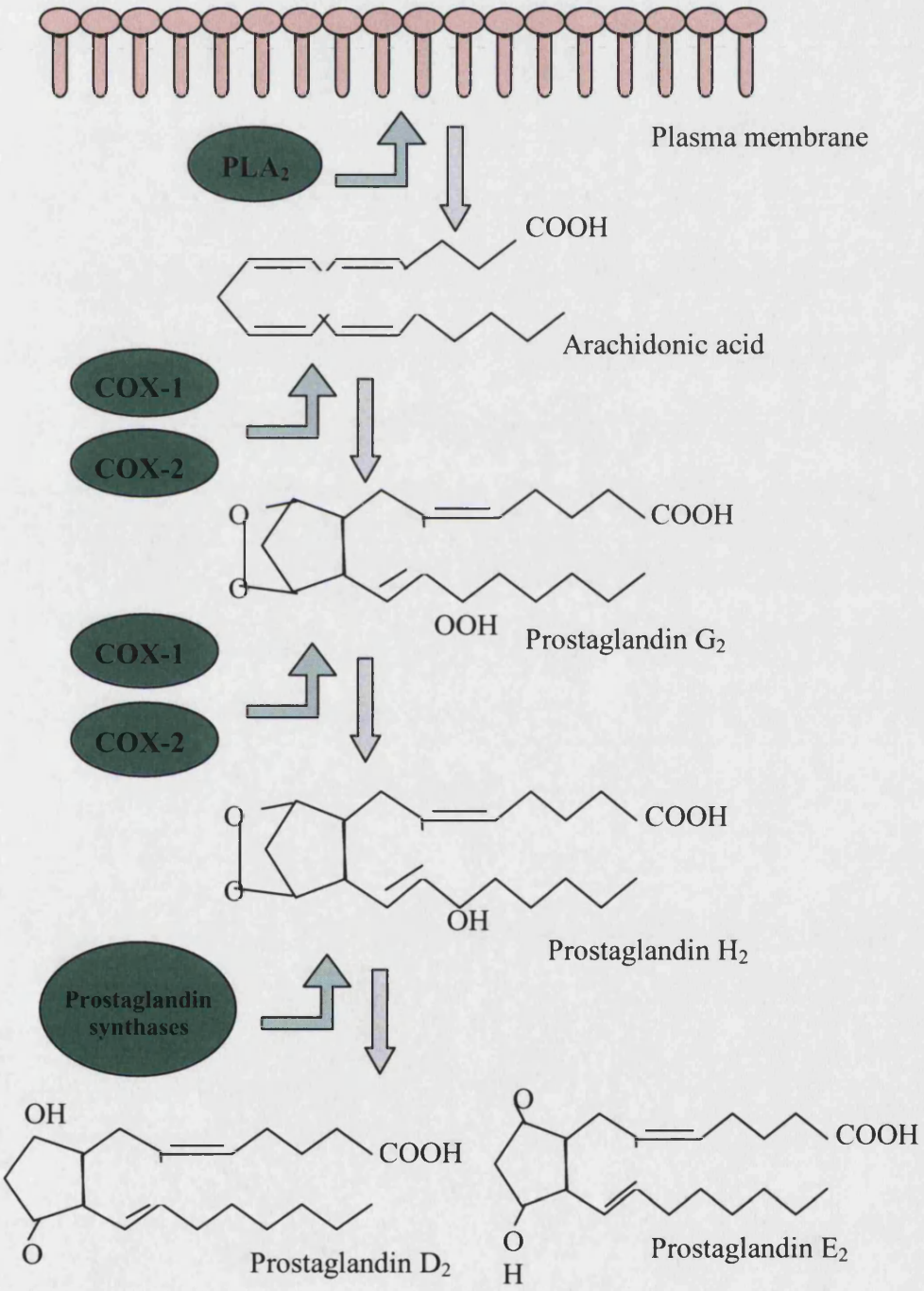
Despite the popularity of the NSAIDs, their use is associated with unwanted side effects the most common of which being damage to the mucosa of the upper gastrointestinal tract (Cichoz and Celinski, 2002). In a prospective study, 13 of every 1000 patients with rheumatoid arthritis who took NSAIDs for 1 year had serious gastrointestinal complication (Singh and Triadafilopoulos, 1999). The discovery that the COX-2 isoform was expressed in cells exposed to pro-inflammatory stimuli including cytokines, mitogens and endotoxin (O'Banion, 1992, Xie, 1992) led to the postulation that the inhibition of COX-1 led to the unwanted side effects of NSAIDs, whereas inhibition of COX-2 may lead to the desired anti-inflammatory effects, a hypothesis supported by the *in vitro* findings of Warner *et al.,* who comparing >40 NSAIDs and COX-2 selective inhibitors demonstrate an alignment between COX-1 inhibition and gastrointestinal complications (Warner *et al.,* 1999). As most NSAIDs inhibit both COX-1 and COX-2 (Mitchell *et al.,* 1993), this hypothesis served as the basis for the development of a number of COX-2 specific inhibitors. In spite of the similarities in crystallographic structures, the two COX isoforms have been found to have different selectivities for fatty acids and COX inhibitors which, in part, is attributable to COX-2 having a more accommodating cyclooxygenase site as a result of the presence of a valine residue at position 509 instead of the isoleucine present at position 523 in COX-1 (Lecomte *et al.,* 1994; Luong *et al.,* 1996; Kurumbail *et al.* 1996). COX-2 specific inhibitors exploit this lateral extension of the cyclooxygenase site (Reviewed in Fitzgerald, 2003). COX-2 selective inhibitors have proved to be effective analgesics. In 2002 four selective inhibitors of COX-2 – rofecoxib (*Vioxx*), celecoxib (*Celebrex*), parecoxib (*Dynastat injection*) and etoricoxib (*Arcoxia*) were launched in the UK (NHS National prescribing centre, 2002). At present only two phase III clinical trials have

investigated whether COX-2 specific inhibitors alter the incidence of serious gastrointestinal adverse events when compared to NSAIDs. One trial, the Celecoxib Long-term Arthritis Safety Study (CLASS), reported no significant difference in gastrointestinal endpoints between patients receiving celecoxib, diclofenac or ibuprofen (Juni *et al.,* 2002), whereas a separate trial, the Vioxx Gastrointestinal Outcomes Research (VIGOR) study, reported that the incidence of adverse events was reduced from 4% to 2% in patients receiving rofecoxib compared with naproxen (Bombardier *et al.,* 2000). To date, the VIGOR study is the only gastrointestinal-outcomes study to reveal a significant difference between a selective COX-2 inhibitor and an NSAID. However, patients with symptomatic ulcers were excluded from these trials as in experimental animals COX-2 has been demonstrated to play an important role in gastric mucosal defense, its inhibitions delaying healing of chronic gastric ulcers (Peskar *et al.,* 2001; Deviere, 2002). Emerging evidence for a role for COX-2 in the release of protective PGs when production by COX-1 has been lost, raises questions about the safety of administration of COX-2 inhibitors. Gilroy *et al.* (1999) report that in carrageein-induced pleurisy in rats, COX-2 protein expression initially peaked at 2 hours associated with maximal $PGE_2$ synthesis. However, at the 48 hour time point there was observed to be a second increase in COX-2 expression, 350% greater than that at 2 hours. Interestingly, this coincided with resolution of inflammation and minimal $PGE_2$ synthesis. In contrast, levels of $PGD_2$ and its metabolite 15deoxy$\Delta^{12-14}PGJ_2$ (15d-$PGJ_2$) were elevated at 2 hours, decreased as the inflammatory response progressed, and increased again at 48 hours. It is becoming increasingly apparent that the role of COX-2 in inflammatory processes is complex and not solely associated with the onset of an inflammatory reaction. COX-2 is expressed during and produces PGs involved in the resolution of inflammation, its inhibition during this period deleterious in that the resolution of inflammation is delayed.

The importance of the PG biosynthetic pathway as a therapeutic target in inflammatory conditions, and evidence that generation of alternative series of prostaglandins by COX-2 may drive the induction or resolution of an inflammatory response, has led to the theory that greater specificity of effect may be obtained by the targeting specific prostaglandin synthases.

**Figure 4 The prostaglandin biosynthetic pathway.**

Arachidonic acid is converted into prostaglandins by the sequential action of enzymes phospholipase $A_2$ ($PLA_2$), cyclooxygenases -1 and –2 (COX-1 and COX–2) and specific prostaglandin synthases. Green arrows denote enzyme action whereas purple arrows indicate product of enzyme activity.



### 1.2.3 The Terminal Prostaglandin Synthases

The prostaglandin metabolic pathway consists of the three major steps: the liberation of AA from the lipid membrane, the production of $PGH_2$ via the action of the cyclooxygenases, and finally the conversion of $PGH_2$ into a number of bioactive PGs by their respective synthases. These specific PG synthases have different structures and exhibit cell- and tissue-specific localisation (Ueno *et al.*, 2001).

$PGE_2$ is the most common prostanoid and exhibits potent and diverse bioactivities. PGES activity has been detected in both the cytosolic and microsomal fractions of various cells and in most, if not all cases, is glutathione dependent (Murakami *et al.*, 1998). Current evidence suggests that mammalian cells contain at least three forms of prostaglandin E synthase (PGES), mPGES-1 that acts in concert with COX-2 (Murakami *et*, 2000), cPGES that is preferentially coupled to COX-1 (Tanioka *et al.*, 2000), and mPGES-2 a recently identified PGES isoform that acts downstream of both COX-1 and –2 (Murakami *et al.*, 2003). mPGES-1 belongs to the Membrane-Associated Proteins involved in Eicosanoid and Glutathione metabolism (MAPEG) superfamily, and was originally identified as Microsomal GST-Like 1 (MGST-L1) (Jakobsson, *et al.*, 1999). Murakami *et al.* (2000) report that, while not constitutively expressed, mPGES-1 expression may be induced in the lung, stomach, kidney and brain by pro-inflammatory mediators including, LPS, IL-1 and TNF. This study also reports that mPGES-1 is functionally coupled to COX-2 and is sensitive to inhibition by dexamethasone. These findings are supported by those of Stichtenoth *et al.*, who, in rheumatoid synoviocytes, observe a coordinate upregulation of COX-2 and mPGES-1 protein expression 24 hours after incubation with IL-1 or TNF that may be completely blocked by dexamethasone (Stichtenoth *et al.*, 2001). Additionally, Kamei *et al.* report that overexpression of mPGES-1 accelerates $PGE_2$ production and cell proliferation in human colorectal adenocarcinoma (HCA-7) cells, and that co-transfection of HEK293 cells with COX-2 and mPGES-1 resulted in transformation manifested by tumour formation when sub-cutaneously implanted into nude mice (Kamei *et al.*, 2003). These studies collectively underline the potential role of mPGES-1 as a therapeutic target.

In contrast, cPGES is a cytosolic protein constitutively expressed in a wide variety of cells and tissues and is associated with heat shock protein 90 (Hsp90) (Tanioka *et al.*,

2000). Identical to p23, a putative chaperone molecule, cPGES is also thought to be involved steroid hormone receptor stabilisation (Sullivan *et al.,* 1997) and signalling (Freeman *et al.,* 2000), however the precise cellular functions of p23 remain obscure. cPGES is preferentially coupled to COX-1 promoting the immediate conversion of COX-1 derived $PGH_2$ into $PGE_2$ required for tissue homeostasis and does not appear to be upregulated by pro-inflammatory stimuli (Tanioka *et al.,* 2000). Recently, Murakami *et al.* have described a third PGES, mPGES-2. mPGES-2 is a constitutively expressed protein and does not appear to be upregulated by pro-inflammatory stimuli. In contrast to cPGES and mPGES-1, mPGES-2 is able to convert both COX-1 and COX-2 derived $PGH_2$ into $PGE_2$ in both the immediate and delayed response (Murakami *et al.,* 2003). The role of this PGES isoform in health and disease is yet to be defined, although overexpression of mPGES-2 in human colorectal adenocarcinoma cells implies that this enzyme may play a role in tumorigenesis (Kamei *et al.,* 2003).

Prostaglandin D synthase (PGDS) catalyses the isomerisation of $PGH_2$ to $PGD_2$. There are two distinct types of PGDS: lipocalin- type PGDS (L-PGDS) a secretory enzyme abundantly present in the central nervous system, male genitals and heart (Urade *et al.,* 2004), and hematopoietic PGDS (H-PGDS), the only mammalian member of the σ class of glutathions S-transferases, a cytosolic enzyme expressed in mast cells, antigen presenting cells, and Th2 cells (Kanoka and Urade, 2003). Activity of PGDS enzyme is reported to be biphasic, acting in concert with only COX-1 in the immediate phase and only COX-2 in the delayed phase (Murakami *et al.,* 1994). Conversion of $PGH_2$ to $PGI_2$ is catalysed by PGI synthase (PGIS), this PG synthase belongs to the cytochrome-P-450 family and is reportedly localised in the endoplasmic reticulum (ER) and perinuclear membranes coupling with COX-2 in response to LPS (Brock *et al.,* 1999).

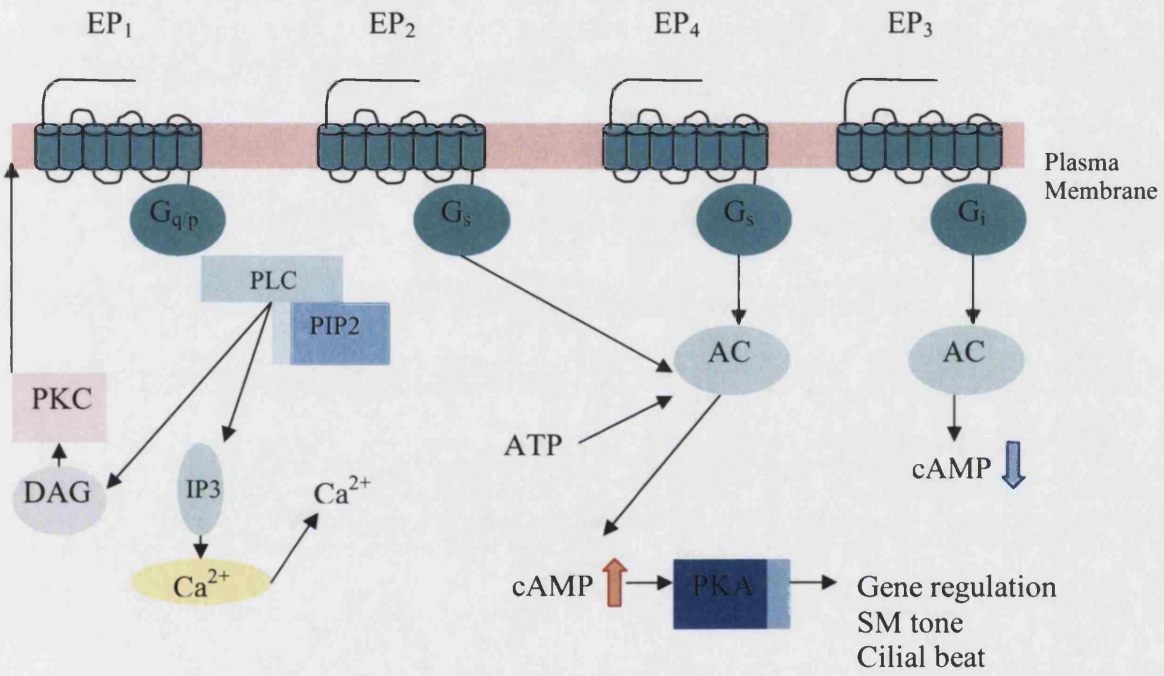
These studies suggest that the prostaglandin synthases may be put into three categories in terms of their localisation and COX preference: 1) perinuclear enzymes preferentially linked to COX-2. This group includes mPGES-1, PGIS and the thromboxane $A_2$ synthase (TXS) and provokes much interest as a therapeutic target for inflammation and carcinogenesis, 2) Cytosolic enzymes that prefer COX-1 such as cPGES and 3) Translocating enzymes that utilise both COX-1 and COX-2 depending upon stimulus, this group includes mPGES-2 and PGDS (Ueno *et al.,* 2001).

### 1.2.4 The prostaglandins

The prostaglandins (PGs) are important autacoids that exert diverse physiological and pathophysiological effects on almost every tissue and system in the body, these effects include modulation of neuronal activity, regulation of ion and water transport, gastrointestinal motility and secretion, and relaxation and contraction of smooth muscle (Wright *et al.,* 2001). In response to a diverse range of stimuli, tissues form and release PGs. As PGs are either chemically or metabolically unstable, for example they are inactivated during a single passage through the lung, it is thought that they only act locally near to their tissue of origin (Reviewed in Narumiya and Fitzgerald, 2001). Derived from AA, the PGs contain a cyclopentane ring and two side chains named α and ω. According to modifications of the cyclopentane ring, the prostaglandins are classified into types A to J. There are four major physiological prostaglanndins $PGE_2$, $PGD_2$, $PGI_2$ and $PGF_{2\alpha}$, each exerting its effect by coupling to distinct G-protein coupled receptors. Furthermore, $PGE_2$ and $PGD_2$ are dehydrated in biological fluids containing serum albumin to yield the cyclopentenone prostaglandins, $PGA_2$ and $PGJ_2$. PGs B and C are not believed to be naturally occurring but rather are produced artificially during extraction procedures (Narumiya *et al.*, 1999).

One of the most studied prostaglandins is $PGE_2$. $PGE_2$ stereospecifically exerts potent tissue specific effects regulating key responses in the major human systems and playing an integral role in a multitude of infections and diseases (Serhan and Levy, 2003). The biological actions of $PGE_2$ are attributed to result from its interaction with specific plasma membrane receptors. Four $PGE_2$ receptor subtypes have been classified and cloned, designated as $EP_1$, $EP_2$, $EP_3$, and $EP_4$, the $EP_3$ and $EP_4$ isoforms also being functionally expressed in the nuclear envelope (Bhuttacharaya *et al.,* 1999). Each EP receptor is coupled with a different second messenger signalling pathway (Figure 5) (Harris *et al.,* 2002).

**Figure 5** Prostaglandin $E_2$ receptor signalling pathways: $EP_1$ is coupled to G-protein $G_{q/p}$ and ligand binding results in an increase in the level of intracellular calcium. $EP_2$ and $EP_4$ are coupled to $G_s$ proteins and induce the expression of cAMP which leads to gene regulation. $EP_3$ is coupled to $G_i$ and is predominantly inhibitory to cAMP. Abbreviations: AC, adenylate cyclase; DAG, diacylglycerol; $IP_3$, inositol trisphosphate,; PIP2, phosphatidylinositol diphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.




$PGE_2$s biological actions are tissue- and organ-specific. In the immune system the actions of $PGE_2$ are generally thought to be suppressive, inhibiting macrophage production of pro-inflammatory cytokines (Nataraj *et al.*, 2001). Enhanced COX-2 expression has been noted in many cancers including cancers of the breast, colon and prostate (Reviewed in Harris *et al.*, 2002). Subsequently, $PGE_2$ synthesis has been observed to be elevated in tumour tissues compared to normal tissues and is linked to tumour-cell survival. It is thought that $PGE_2$ contributes to tumour-cell survival by inhibition of apoptosis and induction of cell proliferation (Sumitani *et al.*, 2001). Furthermore, $PGE_2$ acts as in immunomodulator promoting humoral and Th2-type immune responses that do not favour tumour destruction, and inhibiting Th1-type responses that do favour tumour destruction. The effects of $PGE_2$ in the airways are

diverse and often conflicting and will be discussed detail in the next section. The myriad of effects of $PGE_2$ in health and disease have been well documented, however, the exact mechanisms by which these actions are exerted is poorly understood. Studies using EP receptor knockout mice have contributed to the elucidation of the role of individual receptors in physiological and pathophysiological processes (Table 2) (Reviewed in Narumiya and Fitzgerald, 2001).

**Table 2:** Major phenotypes of mice deficient in EP receptors

| Disrupted gene | Phenotype of knockout | Reference |
|---|---|---|
| **$EP_1$** | Decreased aberrant foci formation in response to carcinogen azoxymethane | Watanabe *et al.*, 1999 |
| **$EP_2$** | Impaired ovulation and fertilisation | Kennedy *et al.*, 1999 |
| | Salt-sensitive hypertension | Kennedy *et al.*, 1999 |
| | Vasopressor or impaired vasodepressor response to intravenous $PGE_2$ | Audoly *et al.*, 1999 |
| | Loss of bronchodilation in response to $PGE_2$ | Fleming *et al.*, 1998 |
| | Impaired osteoclastogenesis *in vitro* | Li *et al.*, 2000 |
| **$EP_3$** | Impaired febrile response to pyrogens | Ushikubi *et al.*, 1998 |
| | Impaired duodenal bicarbonate secretion and mucosal integrity | Takeuchi *et al.*, 1999 |
| | Enhanced vasodepressor response to intravenous infusion of $PGE_2$ | Audoly *et al.*, 1999 |
| | Disappearance of indomethacin-sensitive urine diluting function | Fleming *et al.*, 1998 |
| **$EP_4$** | Patent ductus arteriosa | Nguyen *et al.*, 1997 |
| | Impaired vasodepressor response to intravenous infusion of $PGE_2$ | Audoly *et al.*, 1999 |
| | Decreased inflammation and bone resorption | Miyaura *et al.*, 2000 |

$PGD_2$ has been demonstrated to bind to and activate two G-protein coupled receptors, CRTH2 (Chemoattractant Receptor-homologous molecule expressed on T-Helper type 2 cells) (Sawyer *et al.,* 2002) and DP (Boie *et al.,* 1995). Functional studies have revealed that $PGD_2$ activation of CRTH2 results in a G-protein $G_{\alpha i/o}$ dependent, pertussis toxin-sensitive, decrease in intracellular cAMP (Sawyer *et al.,* 2002), whereas the activation of the DP receptor by $PGD_2$ leads to stimulation of adenylate cyclase activity and an increase in intracellular cAMP levels in a G-protein $G_{\alpha s}$-dependent manner (Boie *et al.,* 1995). Through the DP receptor $PGD_2$ has been implicated in different physiological processes including sleep induction (Matsumura *et al.*, 1994) mucus production and allergic responses (Matsuoka *et al.,* 2000), and cell survival (Gervais *et al.*, 2001). The physiological role of CRTH2 is less well characterised. However, it has been demonstrated that its activation by $PGD_2$ can increase Th2 cell motility (Hirai *et al.,* 2001) and modulate eosinophil morphology, motility and degranulation (Gervais *et al.,* 2001).

$PGI_2$ is a potent vasodilator, antithrombotic and antiplatelet agent that mediates its effects through ligation with the IP receptor and activation of adenylate cyclase via $G_s$ (Reviewed in Smyth and Fitzgerald, 2002). Using carragageenan-induced paw swelling and acetic acid-induced writhing as models for acute inflammation and pain in IP receptor deficient mice, Murata *et al.* demonstrate that $PGI_2$ acting on the IP receptor is a critical mediator of these responses (Murata *et al.,* 1997). $PGF_2$ binds to and activated the $G_q$ coupled FP receptor, resulting in activation of PLC. $PGF_2$ and its receptor are implicated in many aspects of reproductive functions and play an important role in parturition. (Narumiya *et al.,* 1999).

The PGs are important therapeutic targets and elucidation of tissue- and receptor subtype-specific effects may lead to new therapies for cancers and inflammatory disorders.

### 1.2.5 Prostaglandins in airway inflammation and cystic fibrosis

PGs are produced under both physiological and pathophysiological conditions by all cell types in the airways and modulate various airway functions including, airway vascular tone, cell proliferation, plasma exudation, inflammatory cell recruitment and activity, cytokine release, mucus secretion and parasympathetic and sensory nerve function (Belvisi *et al.,* 1997). Numerous studies have suggested that airway inflammatory cells produce pro-inflammatory eicosanoids including $PGD_2$ and $PGF_{2\alpha}$, whereas airway epithelial cells play a role in dampening the inflammatory response by producing $PGE_2$ and $PGI_2$ (Pitt *et al.,* 1998 and references therein). However, it is apparent that PGs may have either pro- or anti-inflammatory effects dependent upon the time point during inflammation, the receptor subtypes activated, and the model being observed.

COX-2 expression may be induced, in airway cells, by a number of pro-inflammatory cytokines including TNFα, IL-1 and IFNγ (Mitchell *et al.,* 1994; Belvisi *et al.,* 1997; Pang *et al.,* 1998; Bonnazzi *et al.,* 2000), whereas cytokines IL-4, IL-13 and IL-10 can inhibit COX-2 induction (Berg *et al.,* 2001 and references therein). IL-10 is a cytokine with potent anti-inflammatory activity blocking synthesis of TNFα, IFNγ and IL-8 by human monocytes (de Waal-Malefyt *et al.,* 1991). Interestingly, Bonfield *et al.* demonstrate that production of IL-10 by the CF airway epithelium is significantly lower than in normal controls (Bonfield *et al.,* 1999). In the airway epithelium COX-2 is constitutively expressed and associated with the production of $PGE_2$ (Watkins *et al.,* 1999). As the airway epithelium is a first line of defense against inhaled pathogens and irritants, constitutive expression of COX-2 may be a consequence of continuous exposure to low-level environmental stimuli. Elevated levels of pro-inflammatory cytokines coupled with decreased levels of anti-inflammatory cytokines in the airways favours expression of COX-2 and $PGE_2$ production which in CF are thought to contribute to the inflammatory response and ultimately lung deterioration.

$PGE_2$ is the most common PG in the airways and is predominantly a COX-2 product that has complex effects on airway tone. $PGE_2$ induces bronchodilation by activating $EP_2$ receptors on airway smooth muscle and, conversely, may indirectly induce constriction through ligation of $EP_1$ and $EP_3$ receptors and activation of neural pathways

(Tilley *et al.,* 2003). Furthermore, at high concentrations $PGE_2$ may induce constriction of smooth muscle through agonism at the thromboxane receptor (Reviewed in Pang *et al.,* 1998). Bradykinin (BK) is a potent inflammatory mediator in patients with bronchial asthma inducing airway smooth muscle cells to produce cytokines and express COX-2. Pang and Knox state that $PGE_2$ mediates BK induction of IL-8, a potent chemoattractant for pro-inflammatory cells (Pang and Knox, 1998; Rodgers *et al.,* 2002). Furthermore, $PGE_2$ can synergise with IL-8 to enhance neutrophil migration (Smith *et al.,* 2002). In CF, BK modification of chloride conductance has been attributed to activation of adenylate cyclase by AA cyclooxygenase products. This is supported by studies performed in immortalised CF and control cell lines that demonstrate BK induces higher AA release from cells expressing the ΔF508 CFTR mutation than the control cell line, this may be a result of dysregulation of $PLA_2$ stimulation (Levistre *et al.,* 1993).

*Pseudomonas aeruginosa* causes lethal lung infections in individuals with CF. The lethality of the infection is directly related with inflammation and lung tissue destruction. Smith *et al.* report that homoserine lactone *N*-(3-oxododecanoyl) produced by *Pseudomonas aeruginosa* activates transcription factor NF-κB resulting in induction of COX-2 expression and $PGE_2$ production in human lung fibroblasts and postulate that $PGE_2$ contributes to *Pseudomonas* induced lung pathology (Smith *et al.,* 2002). Furthermore, upregulation of COX-2 expression, through activation of NF-κB, in response to lipoteichoic acid produced by *Staphylococcus aureus* has been observed in human pulmonary epithelial cells, providing further evidence that COX-2 and $PGE_2$ are involved in airway inflammation (Lin *et al.,* 2001). As previously mentioned, in CF an imbalance in the ionic and/or osmotic balance of the airway surface liquid (ASL) results in clogging of the airways with thick, sticky mucus facilitating subsequent colonisation by bacteria such as *Pseudomonas aeruginosa* (Bals *et al.,* 1999). $PGE_2$ is postulated to influence control of electrolyte movement and mucus secretion co-ordinating with intracellular cAMP and calcium to stimulate modifications in the carbohydrate structure of glycoproteins during secretion of chloride, bicarbonate-chloride exchange, and movement of water through exocrine tissues. Disturbed metabolism of $PGE_2$ in CF is thought to inhibit these changes and may contribute to the production of secretions that are dry and difficult to clear from the airways (Anderson, 1984).

Freedman *et al.* report the presence of a membrane lipid defect in the lung, pancreas and ileum from *cftr*$^{-/-}$ mice, characterised by elevated levels of phospholipid- bound AA and a decrease in phospholipid-bound docosahexaenoic acid (DHA). Furthermore, the levels of TNFα, $PGF_{2\alpha}$ and $PGE_2$ in BAL fluid from *cftr*$^{-/-}$ mice were elevated when compared with those in wild-type controls (Freedman *et al.*1999; Freedman *et al.*, 2002). These findings are supported by those of Zakrzewski *et al.* and Strandvik *et al.* who report that PG levels in sputum, and metabolites of PGs excreted in the urine of CF patients are increased when compared to healthy individuals and may contribute to the pathophysiology of the disease (Zakrzewski *et al.*, 1987 Strandvik *et al.*, 1996). Increased levels of COX-2 and prostanoids, in particular $PGE_2$, are also reported in the BAL fluid from asthmatic patients and cultured epithelial cells derived from asthmatic patients (Taha *et al.*, 2000; Redington *et al.*, 2001).

As previously stated, the PGs are products of COX enzymes, however, it is also reported that PGs participate in the regulation of COX-2 expression and subsequently modulate further PG production. Faour *et al.* observed that, in human synovial fibroblasts, the magnitude and duration of the induction of COX-2 mRNA, protein, and $PGE_2$ production induced by IL-1β was primarily the result of $PGE_2$ stabilisation of COX-2 mRNA decay and protein (Faour *et al.*, 2001). Furthermore, Poligone and Baldwin demonstrate that $PGE_2$ promotes the inherent transcriptional activity of the p65/RelA subunit of NF-κB, and strongly synergises with TNFα to promote NF-κB-dependent transcription and gene expression. In contrast, these authors also report that the cyclopentenone PGs can suppress NF-κB activation by inhibition of IκB kinase, providing a molecular rationale to explain the pro-and anti-inflammatory nature of COX-2 (Poligone and Baldwin, 2001). Activation of NFκB is exaggerated in CF bronchial epithelial cells (Venkatakrishnan *et al.*, 2000). Exaggerated NF-κB activation observed in CF may be, to some extent, a result of elevated $PGE_2$ produced by a constitutively expressed and active COX-2 provided with elevated levels of substrate, activated NF-κB further inducing transcription of COX-2 and pro-inflammatory genes such as IL-8 and TNFα, perpetuating the production of PGs.

Accumulating evidence suggests the induction and regulation of COX-2 may be key elements in the pathophysiological process in a number of inflammatory disorders of

the airways such as cystic fibrosis (Pang, 2001). In diseases such as CF where a chronic inflammatory response leads to the destruction of the host tissue, therapeutic control of PG production may be a target to delay deterioration of lung function. However, the constitutive expression of COX-2 by airway epithelium and the ability of $PGE_2$ to exert both pro- and anti-inflammatory actions suggests a homeostatic role for COX-2 products in the airways, therefore long term inhibition of this enzyme may induce serious side effects. Elucidation of the exact mechanisms behind PG induced airway inflammation will hopefully provide better therapeutic approaches to regulating inflammatory disorders of the airways such as asthma and CF.

## 1.3 Peroxisome proliferator activated receptor gamma and ligands

### 1.3.1 Peroxisome proliferator – activated receptors (PPARs)

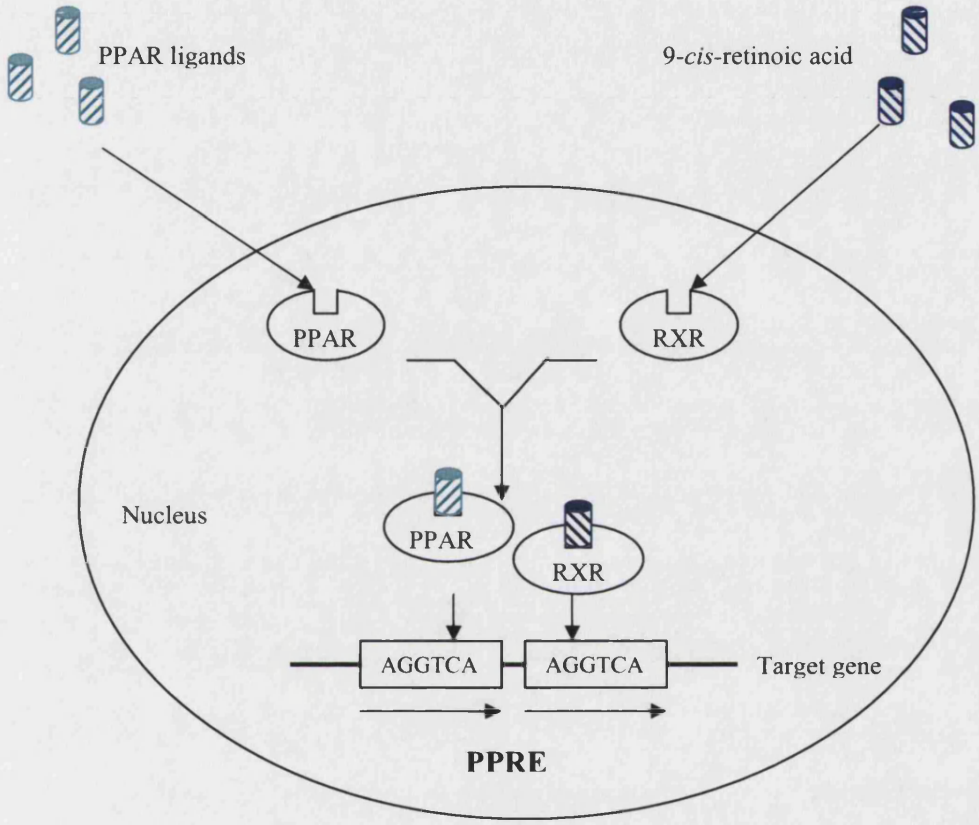
In the 1990s peroxisome proliferator – activated receptors (PPARs) were identified as nuclear hormone receptor transcription factors involved in a number of diseases with complex causes such as diabetes, obesity, atherosclerosis and cancer (Kersten *at al,* 2000). The name PPAR is derived from the identification of one isoform as a target for xenobiotic compounds that can induce proliferation of peroxisomes in the liver (Rosen and Spiegelman, 2001). Three PPAR isoforms (α, β, and γ) have been identified to date. Each isoform is encoded by a separate gene, shows distinct tissue localisation, and is associated with selective ligands (Kliewer *et al.,* 1994; Vamecq and Latrouffe, 1999). All members of this super family have a similar structural organisation. An N-terminal region that allows ligand-independent activation and is negatively regulated by phosphorylation, a DNA binding domain comprising of two zinc fingers separated by a linker region, and the C-terminal ligand binding domain (Murphy and Holder, 2000 and references therein). PPARs regulate gene expression by binding as heterodimers with retinoid -X – receptors (RXRs) that have been activated by the binding of 9-*cis* retinoic acid, to peroxisome proliferator – response elements (PPREs) in the promoter regions of target genes (Figure 6) (Reviewed in Desvergne and Wahli, 1999). PPREs contain the hexameric DNA consensus sequence AGGTCA arranged as a direct repeat spaced by one nucleotide and sequences recognised by the PPAR-RXR heterodimer. It has been recognised that different PPAR isoforms recognise distinct PPREs (Murphy and Holder, 2000 and references therein).

Little is known about the clinical significance of PPARβ although it is implicated in colon cancer and is linked to basic lipid metabolism (Basu-Modak *et al.,* 1999). However, more is known about the physiological roles of PPARα and PPARγ. PPARα is highly expressed in the liver, heart, proximal tubules of the kidney and intestinal mucosa which exhibit high catabolic rates of fatty acids (Braissant *et al.,* 1996). PPARγ exists in two isoforms 1 and 2. PPARγ-2 is expressed in high levels in adipose tissue, playing a critical role in the differentiation of pre-adipocytes into adipocytes, whereas PPARγ-1 is widely distributed and has been observed to be expressed in the immune system (Mukherjee, 1997), airway epithelium (Michael *et al.,* 1997; Wang *et al.,* 2001),

endothelial cells (Bishop-Bailey, 1999) and vascular smooth muscle (Bishop-Bailey, 2001). Studies have revealed that both natural and synthetic ligands of PPARγ possess anti-inflammatory properties (Serhan, 1996) attenuating the production of macrophage-derived cytokines such as IL-1 and TNFα (Jiang *et al.,* 1998; Ricote *et al.,* 1998; Welch *et al.,* 2003) and regulating the expression of COX-2 and inducible nitric oxide (Coleville-Nash *et al.,* 1998). The down-regulation of inflammatory pathways by PPARγ ligands makes this receptor isoform a potential target for therapies aimed at disorders in which the inflammatory response is elevated or dysregulated. However, studies demonstrating that PPARγ ligands are able to up- regulate COX-2 expression in mammary epithelial cells, colonic epithelial cells (Meade *et al.,* 1999), rabbit corneal epithelium (Bonazzi *et al.*, 2000), and primary human monocytes (Pontsler *et al.,* 2002) and, opposingly, down-regulate COX-2 expression in airway epithelial cell lines (Wang *et al.,* 2001), human astrocytes (Janabi, 2002), mesangial cells (Sawano *et al.*, 2002), and macrophages (Inoue *et al.,* 2000) suggest that the effects of PPARγ may be tissue specific. Elucidation of the tissue specific effects of PPARγ in inflammation is necessary in the development new approaches to therapy for chronic inflammatory diseases based upon the effects of PPARγ ligands.

PPARγ is expressed in several human malignancies including, breast (Mueller *et al.,* 2000), colon (Dubois *et al.,* 1999) and lung (Tubouchi *et al.,* 2000). Recent reports suggest that both natural and synthetic agonists of PPARγ can have anti-proliferative effects and induce apoptosis and terminal differentiation of a number of different transformed and cancerous cell lines (Reviewed in Na and Surh, 2003). However, a number of studies also demonstrate that PPAR ligands suppress apoptosis and stimulate G1-arrested cells to re-enter the cell cycle and induce DNA synthesis (Keller *et al.,* 2000 and references therein). The different effects of PPAR ligands on apoptosis may depend on the PPAR subtype activated or the cell line observed.

**Figure 6:** Activation of PPARs, by binding of PPAR ligands, leads to heterodimerisation of the PPAR with activated RXR. Subsequently the receptor heterodimer can bind to PPREs in the promoter region of target genes, such as COX-2, and regulate transcription in a tissue specific manner.



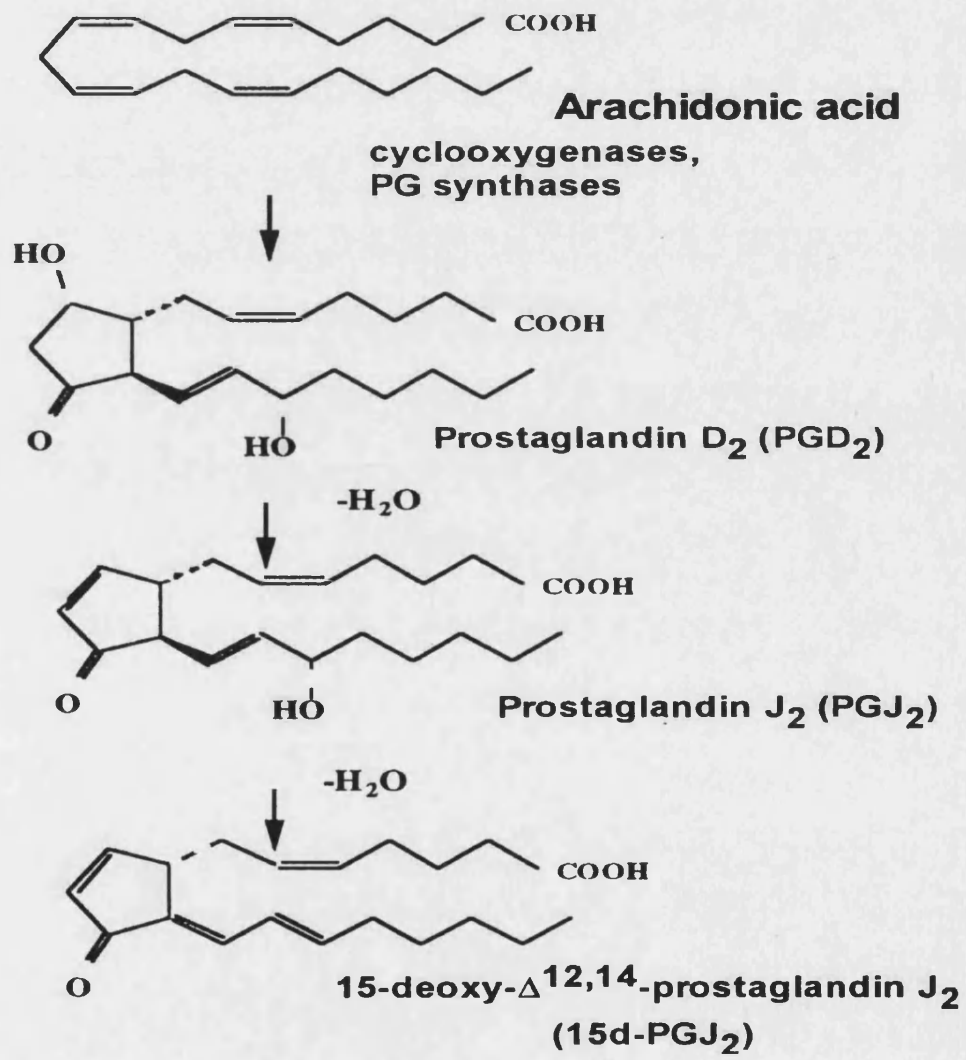

### 1.3.2 PPARγ ligands

As PPARγ plays a role in many physiological and pathophysiological pathways, there has been intense interest in identifying an endogenous, high-affinity ligand for this receptor. In 1995 Forman *et al.* and Kliewer *et al.* independently reported that 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (15d-$PGJ_2$) activates PPARγ (Forman *et al.*, 1995; Kliewer *at al,* 1995). 15d-$PGJ_2$ was first identified in 1983 as a degradation product of $PGD_2$, formed after incubation for extended periods of time in the presence of albumin (Fitzpatrick and Wynalda, 1983). $PGD_2$ undergoes chemical dehydration, losing a molecule of water to form $PGJ_2$, $PGJ_2$ then undergoes further dehydration, losing the 15-hydroxyl group, in

addition to migration on the 13,14-double bond of $PGJ_2$ to form 15d-$PGJ_2$ (Figure 7) (Reviewed by Powell, 2003).

**Figure 7 Prostaglandin $D_2$ Metabolism**

Dehydration of $PGD_2$ to 15-d$PGJ_2$



A number of studies have reported 15d-$PGJ_2$ inhibits inflammatory cytokine production and macrophage activation (Jiang *et al.,* 1998; Ricote *et al.,* 1998; Hinz *et al.,* 2003). However, 15d-$PGJ_2$ has been shown to induce responses in cells that do not express PPARγ (Jiang *et al.,* 1998; Chawla *et al.,* 2001). Therefore, role of 15d-$PGJ_2$ in inflammation is thought to be attributable not only to its binding and activating PPARγ, but to its modulation of the activity of the transcription factors NF-κB and AP-1 (Castrillo *et al.,* 2000; Straus *et al.,* 2000), activation of H-Ras (Oliva *et al.,* 2003), activation of MAP kinase cascades (Lennon *et al.,* 2002; Jung *et al.,* 2003), reactive oxygen species (ROS) generation (Lennon *et al.,* 2002) and activation of cell membrane $DP_2$ receptors (Monneret *et al.,* 2002). Concentrations of 15d-$PGJ_2$ required to activate PPARγ or ligate proteins in the NF-κB signalling pathway *in vitro* range from 2.5 – 100μM (Castrillo *et al.,* 2000; Narumiya and Fitzgerald, 2001) whereas, *in vivo,* biologically active concentrations of conventional exogenous PGs are observed to be in the picomolar range (Fitzgerald, 1981). Using a highly sensitive liquid chromatography / tandem mass spectrometry for 15d-$PGJ_2$, Bell-Parikh *et al.* report that in 3T3-L1 fibroblasts, COX-2 produces low levels of 15d-$PGJ_2$ (~2pM) and that these levels are lower than the amounts required for PPARγ dependent differentiation. Levels of 15d-$PGJ_2$ detected in urine and synovial fluid were also too low to be an endogenous activator of PPARγ, and its biosynthesis is unaltered in inflammation *in vivo* (Bell-Parikh *et al.,* 2003). As levels of 15d-$PGJ_2$ produced *in vivo* (pM) are significantly lower than required for modulation of responses and activation of PPARγ *in vitro* (μM), this study throws into question the relevance of the physiological role of 15d-$PGJ_2$. However, further investigation into the tissue specific production and effects of 15d-$PGJ_2$ is necessary.

Thiazolidinediones (TZDs) are a class of synthetic compounds that potentiate insulin action in target tissues, alleviate hyperglycemia and are efficacious in treating type II diabetes (Reviewed in Olefsky, 2000). Additional roles for TZDs were suggested following the discovery that, at the molecular level, these compounds function as highly specific ligands of PPARγ (Lehmann *et al.,* 1995). TZDs include troglitazone, rosiglitazone and ciglitazone. Troglitazone has been shown to inhibit inflammatory cytokine production and macrophage activation (Jiang *et al.,* 1998; Ricote *et al.,* 1998) Despite the TZDs having a binding affinity for PPARγ in the nanomolar range (Jiang *et*

*al.,* 1998), the concentrations of troglitazone used to elicit these responses was in the micromolar range. Additionally, evidence that TZDs can inhibit TNFα and IL-6 secretion by 60 – 80 % in PPARγ deficient macrophages indicates that, as with 15d-$PGJ_2$, the anti-inflammatory effects of TZDs may be PPARγ independent (Jiang *et al.,* 1998).

NSAIDs such as indomethacin are accepted to exert their clinical activity by inhibiting COX, thereby blocking the production of PGs. However, evidence that indomethacin blocks adipocyte differentiation and induces terminal differentiation of preadipocyte cell lines at concentrations 2 – 3 orders of magnitude higher than those required for inhibition of COX (Knight *et al.,* 1987), suggests that the effects of NSAIDs such as indomethacin may not solely be attributable to COX inhibition. In 1997 Lehmann *et al.* reported that indomethacin was a micromolar ligand for PPARγ and provided evidence that the adipogenic actions of this NSAID were a result of its binding to and activating PPARγ (Lehmann *et al.,* 1997). It is becoming clear that the emerging role of NSAIDs, such as aspirin and sulindac in the prevention and treatment of human cancer (Duperron and Castonguay, 1997) is a consequence not only of COX inhibition, but the ability of NSAIDs to affect alternative cellular targets. This is supported by the findings of Wick *et al.,* who report that both sulindac sulfide and ciglitazone stimulate a promoter construct containing a PPARγ response element linked to luciferase and are able to inhibit the growth of non-small-cell lung cancer cell growth. This study also demonstrates that overexpression of PPARγ strongly inhibits the transformed growth properties of the cells (Wick *et al.,* 2002).

In addition to agonists, a small number of PPARγ antagonists have been described. Bisphenol A diglycidyl ether (BADGE), an antagonist that binds with micromolar affinity for PPARγ, is a synthetic substance used in the production of plastics discovered during a screen for endogenous ligands for this receptor (Wright *et al.,* 2000). Wright *et al.* report that, in 3T3-L1 and 3T3-F442A cells BADGE does not activate the transcriptional activity of PPARγ but antagonizes the ability of ligands, such as rosiglitazone, to activate the transcriptional and adipogenic activity of the receptor (Wright *et al.,* 2000). However, Bishop-Bailey *et al.* report that, in the ECV304 cell line, BADGE acts as an agonist of PPARγ at a similar level to 15d-$PGJ_2$

and ciglitazone inducing transcriptional activity of a PPAR reporter gene and cell death (Bishop-Bailey *et al.,* 2000). These studies, once again, underline the importance of cell type specificity with respect to the effect of PPARγ ligands upon cellular activity.

### 1.3.3 The role of PPARγ in airway inflammation

Wang *et al.* demonstrate that airway epithelial cells express constitutively high levels of PPARγ and postulate that one of its roles in the airways to be that of a negative regulator of inflammation (Wang *et al.,* 2001). This is supported by the findings of Patel *et al.* (2003) who report that PPARγ ligands inhibit cell growth, induce apoptosis and inhibit GM-CSF release in human airway smooth muscle cells. Additionally, this study also demonstrates that the PPARγ ligands 15d-$PGJ_2$ and ciglitazone are more effective at inhibiting cell growth and GM-CSF release than corticosteroids. These data suggest that PPARγ ligands may be useful as effective anti-inflammatory therapies for treatment of diseases such as chronic obstructive pulmonary disease (COPD) and asthma where steroids are ineffective or have deleterious side effects. As previously mentioned, the inhibitory effects of PPARγ ligands on the production of pro-inflammatory mediators are not only attributable to activation of PPARγ, but to modulation of the activity of transcription factors including NF-κB (Castrillo *et al.,* 2000; Straus *et al.,* 2000). It has been reported that 15d-$PGJ_2$ inhibits NF-κB-dependent transcription by two distinct mechanisms. First, 15d-$PGJ_2$ can interrupt NF-kB dependent gene expression through the covalent modification of critical cysteine residues in IKK, preventing IκB degradation and nuclear entry of NF-κB and secondly, 15d-$PGJ_2$ can directly inhibit binding of NF-kB to target DNA (Reviewed in Na and Surh, 2003). In diseases such as CF where activation of NFκB is exaggerated resulting in increased inflammatory mediator production, the use of PPARγ ligands may prove effective in reducing inflammation and airway damage. Expression of PPARγ is augmented in the airway epithelium of asthmatics compared with control subjects, and is negatively related to decline in lung function. Furthermore, corticosteroids have been observed to decrease the level of PPARγ expressed (Benayoun *et al.,* 2001). It is postulated that increased expression of PPARγ in asthmatics may be a result of increased levels of IL-4, a cytokine produced by inflammatory cells with the ability to

up-regulate PPARγ (Huang *et al.,* 1999) and its down-regulation a result of the corticosteroid inhibition of IL-4 production (Bentley *et al.,* 1996). Additionally, the ability of PPARγ ligands to down-regulate the synthesis and release of macrophage-derived cytokines, seen to be elevated in the ASL of CF patients, support a major role for PPARγ in disorders characterised by inflammation (Jiang *et al.,* 1998; Ricote *et al.,* 1998; Welch *et al.,* 2003). However, not all studies demonstrate an anti-inflammatory role for PPARγ in the airways. Pang *et al.* report that in human airway smooth muscle (HASM) cells NSAIDs induce COX-2 expression through PPARγ activation which may intensify the inflammatory response by increasing PG production (Pang *et al.,* 2003).

In addition to its anti-inflammatory effects, 15d-$PGJ_2$ has also been reported to have cytoprotective effects. It has been shown that 15d-$PGJ_2$ can induce expression of the anti-oxidant enzyme Heme-Oxygenase-1 (HO-1) in activated microglial cells (Koppal *et al.,* 2000), murine macrophages (Colville-Nash *et al.,* 1998; Lee *et al.,* 2003), and cardiac myocytes (Wayman *et al.*, 2002). HO-1 is an enzyme that catabolises heme to produce carbon monoxide (CO), free iron and biliverdin. Biliverdin is rapidly converted into the anti-oxidant bilirubin by the enzyme biliverdin reductase and any free iron is sequestered by ferritin (Maines, 1997). Three distinct isoforms of HO have been identified, each the product of a separate gene. HO-1 is an inducible isoform, its expression upregulated by pro-inflammatory cytokines, oxidative stress, heavy metals and UV light, whereas HO-2 and HO-3 are constitutively expressed isoforms. However, to date, HO-3 has only been expressed constitutively in rat neurones (McCoubrey *et al.,* 1997). Several studies have shown that HO-1 has anti-inflammatory properties and the role of HO-1 as an anti-oxidant is well documented. Poss *et al.* demonstrate that HO-1 deficient mice develop a chronic inflammatory state that increases with age (Poss *et al.,* 1997). Furthermore, overexpression of HO-1 by gene transfer into rat lungs provided protection from hyperoxic injury (Otterbein *et al.,* 1999). Elevated levels of CO are found in the exhaled breath of patients with inflammatory diseases such as asthma and CF, the source of which may be the airway epithelium (Donnelly and Barnes, 2001). In models of pulmonary inflammation, HO-1 is potently cytoprotective and it is postulated that the anti-inflammatory effects of HO-1 are in large measure mediated by CO (Reviewed in Otterbein *et al.,* 2003). Lee *et al.*

have demonstrated that HO-1 induced by IL-10 mediates the anti-inflammatory effects of this cytokine (Lee *et al.,* 2003).

Levonen *et al.* have shown that 15d-$PGJ_2$, at low micromolar concentrations, induces a robust increase in cellular reduced glutathione (GSH) (Levonen *et al.,* 2001). GSH is an important component of the cells anti-oxidant defenses. The GSH system effectively scavenges oxidants thereby protecting cells and tissues from damage. GSH production by the respiratory epithelium of CF airways is deficient compared to production by the epithelium of non-CF individuals. It is thought that the production of GSH protects the epithelial surface from oxidants produced by activated macrophages and neutrophils (Roum *et al.,* 1999). Additionally, preliminary findings by Na *et al.* show that doses of 15d-$PGJ_2$ (<5μM) rescue rat phenochromacytoma cells from oxidative or nitrosative stress (Na and Surh, 2003). This is supported by the findings of Colville- Nash *et al.* who report that 15d-$PGJ_2$ induced HO-1 expression is accompanied by a reduction in inducible nitric oxide synthase (iNOS) expression and subsequent NO production (Colville-Nash *et al.,* 1998). In contrast, at higher concentrations (10μM) 15d-$PGJ_2$ induces endothelial cell apoptosis (Levonen *et al.,* 2001).

PPARγ has been implicated in the pathophysiology of diverse human disorders encompassing inflammation, atherosclerosis, diabetes and obesity (Kersten *et al.,* 2000). The ability of PPARγ ligands to inhibit the activity of transcription factors such as NF-κB and down-regulate the production of pro-inflammatory mediators including cytokines, COX-2, and iNOS whilst increasing the expression of protective mediators such as GSH and HO-1 show the potential of PPARγ ligands to target inflammation. These findings are thought to be especially relevant to inflammatory disorders of the airways, such as CF, where pro-inflammatory pathways are up-regulated and anti-inflammatory pathways are suppressed leading to tissue damage and deterioration of lung function. However, the inhibition of multiple cell signalling pathways and contradicting effects of PPARγ in different cell types suggest that the exact mechanisms of effect and tissue specific effects need to be further elucidated.

## 1.4 Aims of the study

Accumulating evidence suggests the induction and regulation of COX-2 may be key elements in the pathophysiological process in a number of inflammatory disorders of the airways such as cystic fibrosis (Pang, 2001). Zakrzewski *et al.,* (1987) and Strandvik *et al.,* (1996) report that PG levels in sputum, and metabolites of PGs excreted in the urine of CF patients are increased when compared to healthy individuals and may contribute to the pathophysiology of the disease. Elucidation of the PG biosynthetic pathway in the human airway epithelium may facilitate better therapeutic approaches to regulating inflammatory disorders of the airways such as asthma and CF. Recent evidence suggests that ligands of the nuclear hormone receptor PPARγ are able down-regulate the production of inflammatory mediators, including COX-2, in the airways (Wang *et al.,* 2001; Patel *et al.,* 2003). Additionally, 15d-$PGJ_2$ has been shown to increase the expression of HO-1 in a number of cell systems. HO-1 expression, in patients with CF, is reported to be cytoprotective and augmentation of its expression is proposed as a potential therapy against bacterial injury (Zhou *et al,* 2004). It is therefore of interest to assess the anti-inflammatory potential of PPARγ ligands in the airway epithelium.

The aim of this project was to investigate whether PPARγ ligands are able to modulate PG production in the human tracheal epithelium using cell lines with a CF phenotype, ΣCFTE29o$^-$, and non-CF phenotype, 9HTEo$^-$.

The objectives of this project were therefore as follows:

- To determine the expression of enzymes of the $PGE_2$ biosynthetic pathway, and their regulation by pro-inflammatory mediators, in human tracheal epithelial cell lines ΣCFTE29o$^-$ and 9HTEo$^-$, cells with a CF and non-CF phenotype respectively
- To characterise the production of PGs by human airway epithelial cells and the regulation of their production by pro-inflammatory cytokines
- To investigate whether the PG biosynthetic pathway can be modulated by PPARγ and its ligands
- To determine the cytoprotective effects of PGs in human tracheal epithelial cell line

# 2. Materials

| | |
|---|---|
| 2- Mercaptoethanol | Sigma, Poole, UK |
| Acrylamide | Bio-Rad, UK |
| Ammonium persulphate | BDH, Poole, UK |
| Aprotinin | Sigma, Poole, UK |
| BADGE | Tocris, Bristol, UK |
| Bovine serum albumin (BSA) | Sigma, Poole, UK |
| Bromophenol blue | Sigma, Poole, UK |
| Cell culture plastics | Nunc, Leicestershire UK |
| cPGES Antibody | Cayman Chemicals, MI, USA |
| COX-1 Antibody | Santa-Cruz, CA, USA |
| COX-2 Antibody | Santa-Cruz, CA, USA |
| Dimethyl sulphoxide (DMSO) | Sigma, Poole, UK |
| Enhanced chemiluminescence detection kit (ECL) | Amersham International, UK |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma, Poole, UK |
| Foetal bovine serum (FBS) | Gibco BRL, Paisley, UK |
| Glycerol | Sigma, Poole, UK |
| Glycine | Sigma, Poole, UK |
| Human recombinant IL-1β | Generous gift from Glaxo Wellcome, Greenford, UK |
| Human recombinant IL-13 | Generous gift from Dr A. Minty, Sanofi Elf Bio Recherches, Laberges, France |
| Human recombinant IFNγ | Boehringer Mannheim, Mannheim, Germany |
| Human recombinant TNFα | Generous gift from Bayer, Slough, UK |
| Hydrochloric acid | BDH, Poole, UK |

| | |
|---|---|
| Hygromycin | Calbiochem, Darmstadt, Germany |
| Leupeptin | Sigma, Poole, UK |
| Methanol | Fisons, Loughborough, UK |
| Molecular weight markers | Biorad, Hertfordshire, UK |
| mPGES antibody | Cayman Chemicals, MI, USA |
| N-(2-(Cyclohexyloxyl)-4-nitrophenyl) methanesulfonamide (NS-398) | Sigma, Poole, UK |
| Ponceau S solution | Sigma, Poole, UK |
| Prostaglandin $E_2$ ELISA kit | R&D Systems, Abingdon, UK |
| Prostaglandin $D_2$ MOX EIA | Cayman Chemicals, MI, USA |
| Sodium azide | Sigma, Poole, UK |
| Sodium chloride | Sigma, Poole, UK |
| Sodium dodecyl sulfate (SDS) | Sigma, Poole, UK |
| Sodium fluoride | Sigma, Poole, UK |
| Sodium hydroxide | Sigma, Poole, UK |
| Sodium molybdate | Sigma, Poole, UK |
| Sodium nitrite | Sigma, Poole, UK |
| Sodium orthovanadate | Sigma, Poole, UK |
| TEMED | Sigma, Poole, UK |
| Tissue culture reagents | Gibco BRL, Paisley, UK |
| Trizma base | Sigma, Poole, UK |
| Trypan blue | Sigma, Poole, UK |
| Tween-20 | Sigma, Poole, UK |
| Versene | Gibco BRL, Paisley, UK |

| | |
|---|---|
| Vitrogen | Gibco BRL, Paisley, UK |

# 3. Methods

## 3.1 Cell culture conditions

### 3.1.1 ΣCFTE29o$^-$ and 9HTEo$^-$ cell lines

A lack of tissue and the need to determine the biochemical and genetic causes of airway disease in CF has led to the development of defined cell culture systems. Primary cell culture has played an important role in the characterisation of cell specific functions, however its use is limited by availability of tissue and the small number of viable cells generated by the available tissue. Additionally, variability between different donor epithelia complicates the design and interpretation of data obtained from primary cells. Development of immortal cultures of airway epithelial cells has provided a useful system in which to study the biochemical defects in CF. The ΣCFTE29o$^-$, a human tracheal epithelial cell line homozygous for the ΔF508 CFTR mutation, and the human tracheal epithelial cell line 9HTEo$^-$ used in this study were generous gifts from D.C. Gruenert (University of San Francisco, CA, USA). These cell lines were created by transfecting human tracheal epithelial cell lines with a plasmid containing a replication-defective simian virus 40 (SV40) genome. The process resulted in transformed human tracheal epithelial cell lines, which have passed through crisis and are immortal whilst retaining aspects of the original phenotype. These include the presence of microvilli, responsiveness to agonists, $Cl^-$ ion transport and the ability to form tight junctions. Furthermore, electrophysiologically, ΣCFTE29o$^-$ cells have been demonstrated to show no cAMP-dependent Cl transport as observed in primary cell lines expressing the ΔF508 mutation (Gruenert *et al.,* 1988, Kunzelmann *et al.,* 1993).

The maintenance of phenotypic aspects of primary cells such as $Cl^-$ ion transport and responsiveness to agonists by the ΣCFTE29o$^-$ and 9HTEo$^-$ cell lines make them useful in the characterisation of airway epithelial biology and airway surface liquid composition in both the presence and absence of defective chloride conductance respectively. In this study these cell lines are used in order to determining the biologic significance of this mutation in the airway epithelium and its effect on prostaglandin production and regulation.

### 3.1.2 A549 Airway Epithelial Cell Line

The human type II Alveolar carcinoma cell line A549 (Lieber *et al.,* 1976) was obtained from the American Type Culture Collection (ATCC).

### 3.1.3 Culture of ΣCFTE29o$^-$ and 9HTEo$^-$ cell lines

ΣCFTE29o$^-$ and 9HTEo$^-$ cells were routinely cultured in Vitrogen coated 170cm$^2$ tissue culture flasks in MEM medium supplemented with 10% (v/v) FBS (referred to as complete medium), penicillin (10 u/ml), streptomycin (10 μg/ml), and fungizone (0.5 μg/ml). Cultures were maintained in a humidified air / 5% $CO_2$ atmosphere. The medium was changed every 2 – 3 days.

A549 cells were routinely cultured in 170cm$^2$ tissue culture flasks in Dulbecco's modified Eagle's medium with sodium pyruvate + L-glucose + pyroxidine (DMEM). The medium was supplemented with 10% (v/v) FBS (referred to as complete medium), penicillin (10 u/ml), streptomycin (10 μg/ml), and fungizone (0.5 μg/ml). Cultures were maintained at 37°C in a humidified air / 5% $CO_2$ atmosphere. The medium was changed every 2 – 3 days.

To subculture confluent monolayers, the medium was removed and the cells washed three times with PBS (w/o $Ca^{2+}$ and $Mg^{2+}$). The cells were then washed with 3 ml Trypsin-EDTA mixture of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA. The cells were incubated at room temperature until the cells had detached from the flask. The action of the Trypsin-EDTA was inhibited by adding 10ml of complete MEM or DMEM medium and the cell suspension was centrifuged at 200g for 10 min. The pellet was resuspended in complete medium and cell counting and viability were checked in a Neubauer haemacytometer after mixing with Trypan Blue (Sigma). Dead cells stained blue, resulting from uptake of Trypan blue. Cell viability was always greater than 95%. Cells were counted and then seeded at 1 – 1.5 x $10^6$ /ml of complete MEM medium, into 170 cm$^2$ tissue culture flasks for further culture, into 92mm petri dishes at 1 x $10^5$ / 3 ml of complete MEM medium, for western analysis and into 24 well plates at 1 x $10^4$ /ml of complete MEM medium, for ELISA experimental protocols. Flasks and plates reached confluency after approximately four days.

For storage, cells were resuspended at 3 x $10^6$ /ml of freeze medium. The freeze medium contained 10% dimethyl sulfoxide (DMSO), 40% FBS and 50% MEM medium supplemented with 40% FBS. The cell suspension was transferred into cryotubes (Nunc) at 1 ml/tube, gradually cooled in vapour phase of liquid nitrogen overnight and then stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted at 37°C in a water bath, resuspended in 15ml of complete medium and centrifuged at 200g for 10 min. Pellets were resuspended in 25ml of complete MEM medium and transferred into 170 $cm^2$ tissue culture flasks.

## 3.2 Experimental Protocol

Airway epithelial cell lines were seeded into and cultured in vessels appropriate to the planned experiment:

| Assay | Culture Vessel | Cell Number Seeded |
|---|---|---|
| Western analysis | 92 mm petri dishes | 1 – 2 x $10^5$ per dish |
| ELISAs | 24 well plates | 1 x $10^5$ per well |
| Glutathione assay | 170 $cm^2$ flasks | 1 x $10^6$ per flask |

Six to Twenty-four hours prior to experimentation confluent monolayers were washed and cultured in FBS free medium and stimulated with appropriate doses of drugs, cytokines, prostaglandin or vehicle control for the times described in the results section. Supernatants were collected for ELISAs and stored at –80°C until assayed, for prostaglandin ELISAs 10 μM indomethacin was added to supernatants prior to freezing. Total RNA and cellular proteins were extracted as described below. Cell counting and viability were routinely checked at the beginning and end of the experiment, by MTT assay, phase microscopy and by trypan blue exclusion, using representative wells. Cell viability was always greater than 95%.

## 3.3 Western Blot Analysis

### 3.3.1 Overview

Western blot analysis is a technique that enables visualisation of relative amounts of specific proteins expressed within cells. Cells are lysed to extract whole proteins that can then be quantified. Whole cell extracts can be run in a current through a polyacrylamide gel separating proteins according to their molecular weight. Proteins can then be transferred to a nitrocellulose membrane that is incubated with a primary antibody specific for the protein to be assessed. A second incubation is then undertaken with a labelled secondary antibody against the primary antibody. The membrane is then washed and the relative amount of labelled protein assessed, typically by a reaction catalysed by a horseradish peroxidase tag on the secondary antibody that can then be visualised.

### 3.3.2 Sample Preparation

Monolayers of airway epithelial cells were stimulated and incubated at 37°C as described. Stimulations were terminated at the appropriate times with aspiration of the supernatant, a rinse with PBS (w/o $Ca^{2+}$ or $Mg^{2+}$) and the addition of ice cold lysis buffer. Cells were solubilised using a cell scraper and the resulting lysates transferred to 1.5 ml eppendorf tubes. The samples were then centrifuged at 12000g for 5 min and the protein containing supernatant transferred to a fresh eppendorf, an aliquot of the each lysate was then removed for subsequent protein assay. A relevant amount of 5 X SDS sample buffer was then added to the lysate and the resulting solution boiled for 10 min. The samples were then frozen at –80°C.

### 3.3.3 Protein assay

Total protein per lysate was determined using the Bio-Rad DC Protein Assay. This assay is based on the Bradford dye-binding procedure. Known concentrations of bovine serum albumin (BSA) diluted in lysis buffer were used as a standard curve. Samples and standards were diluted in 20% Bio-Rad assay in MilliQ water. 200 µl of standard or sample were transferred to a 96 well plate. The plate was read at 595 nm on a Dynatech MR5000 platereader. The protein concentrations were then calculated by linear regression from the standard curve. Equal concentrations of protein could then be loaded onto polyacrylamide gels for western analysis.

### 3.3.4 Separation of Cellular Proteins by Electrophoresis

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecular size. Sodium Dodecyl Suphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (Laemmli, 1970). Proteins were separated by SDS-PAGE using the Bio-Rad Mini Protean II.

Minigels of the appropriate percentage were prepared as described in the appendix. The resolving gel was poured into the gel equipment and overlaid with MilliQ water. After the resolving gel had set, the water was removed and the stacking gel poured on top and set with a 10 or 15 well comb inserted. When the stacking gel was set the comb was removed. ~ 25 μg of protein was loaded per lane alongside a molecular weight marker. Gels were run in running buffer at room temperature at 100V, until the bromophenol blue tracking dye entered the resolving gel. The voltage was then increased to 200V. Gels were run until the blue band reached the bottom of the running gel. Gels were then placed in transfer buffer.

### 3.3.5 Wet Transfer of Proteins to Nitrocellulose

Each gel was placed on top of a nylon pad and 2 pieces of 3MM filter paper and overlaid with nitrocellulose membrane all of which had been dampened with transblot buffer (Boehringer Mannheim). Another two pieces of dampened filter paper and a nylon pad were placed on top of the nitrocellulose membrane and rolled gently to expel air. The sandwich was then placed in a transblot cassette and placed in a transfer tank (Bio-Rad) filled with ice-cold transblot buffer. An additional ice pack was also added to the tank to prevent the apparatus overheating. The transfer was run at 100V for 1 hour. The membrane was the stained with Ponceau S (Sigma) to check for successful transfer, equal loading and determination of the position of the molecular weight markers. The stain was removed by washing the membrane first with distilled water, followed by a 10 min was with Tris buffered saline (TBS).

### 3.3.6 Immunoblotting of Nitrocellulose bound protein

Non-specific protein binding was blocked by incubation of the membrane for 1 h at room temperature in 5% non-fat dried milk protein reconstituted in TBS. Membranes were then incubated for 2 h - overnight on a rocking platform at room temperature, with an appropriate primary antibody diluted in TBS. Subsequent to incubation membranes were washed for two 5 min periods in TBS/Tween and three ten min. periods in TBS prior to a 1 h incubation with the appropriate secondary antibody, followed by washing as described above. Membranes were drained of excess TBS and 1ml of Enhanced Chemiluminescent (ECL) reagent added to their surface for 1 minute before being exposed to X-ray film that was developed using an RGII-Fuji X-ray film developer.

## 3.4 Prostaglandin $E_2$ ELISA

### 3.4.1 Overview

This assay to measure $PGE_2$ concentration uses a kit from R&D Systems (Abingdon, UK). It is based on the competitive binding technique in which PGE2 present in the supernatant competes with a fixed amount of alkaline phosphatase-labelled $PGE_2$ for sites on a mouse monoclonal antibody. During an incubation period, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody that coats the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The intensity of the colour generated is inversely proportional to the concentration of $PGE_2$ in the sample (Figure 8).

### 3.4.2 Assay procedure

Known concentrations of a $PGE_2$ standard were diluted into cell culture medium and used for a standard curve (7.8 pg/ml - 1000 pg/ml). 100 µl of standard or sample were loaded into a microplate coated with goat anti-mouse antibody. To each was added 50 µl of $PGE_2$ conjugate (conjugated to alkaline phosphatase) and 50 µl of $PGE_2$ antibody solution (mouse monoclonal). The plates were covered and incubated for 18 – 24 h at 4°C. After this incubation the wells were aspirated and washed three times with 200 µl of wash buffer using multi-channel pipette. After the last wash the plate was inverted and blotted against clean paper towelling to ensure removal of all liquid remaining in the wells. pNPP substrate (200 µl) was then added to all wells and incubated for 1 h at 37°C. The optical density at 405 nm was then determined using a Dynatech MR5000 plate reader. The PGE2 concentrations were calculated by comparison to the sigmoid standard curve using BioLinx software.

## 3.5 Prostaglandin $D_2$ ELISA

### 3.5.1 Overview

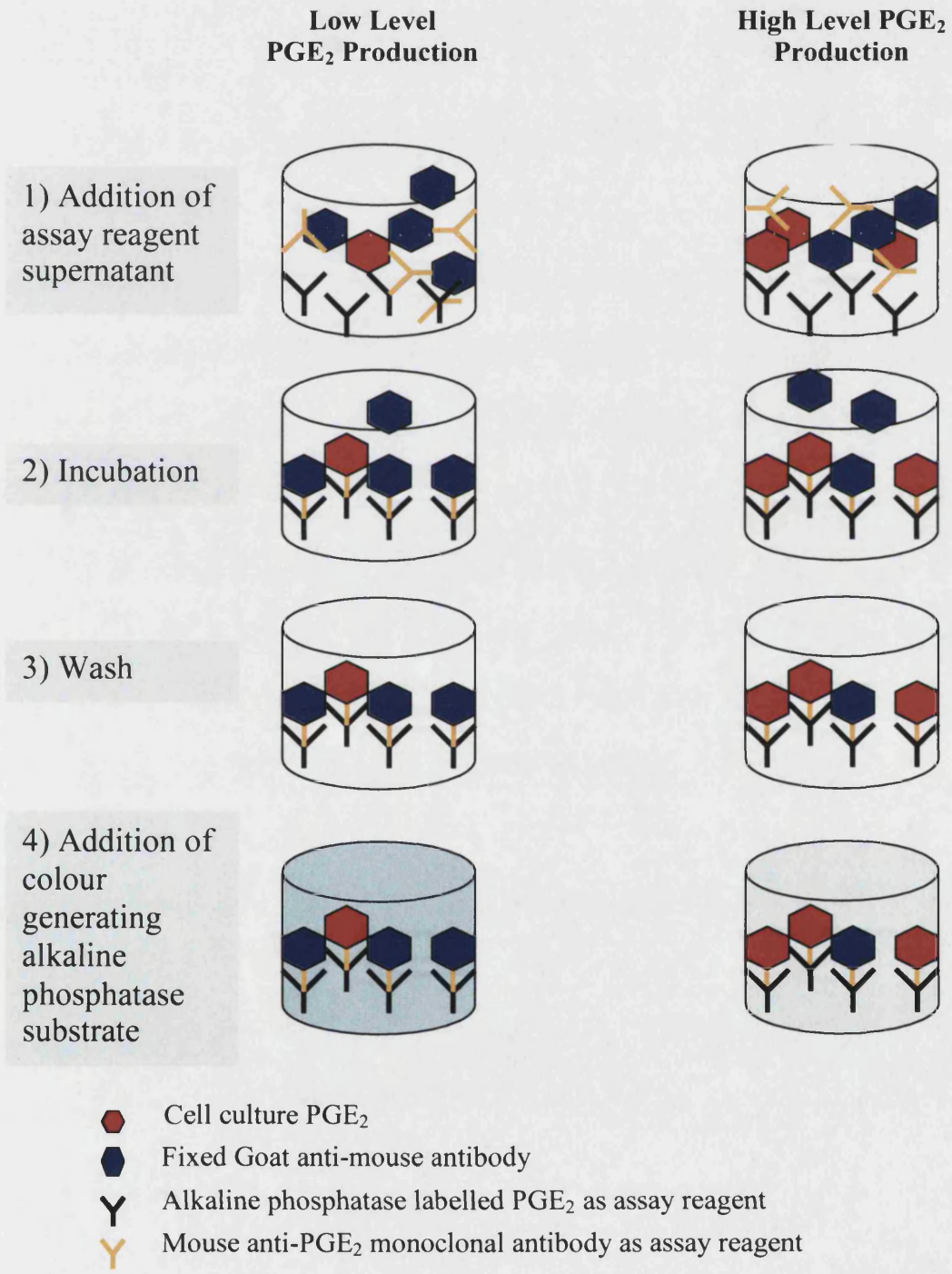
This assay measures $PGD_2$ in cell supernatants and uses a kit from Cayman Chemicals (Ann Arbor, MI, USA). It converts unstable $PGD_2$ to stable $PGD_2$-methoxime ($PGD_2$-MOX) by methoximation. This assay is then based upon the competitive binding technique in which the now present $PGD_2$-MOX competes with a fixed amount of acetylcholinesterase-labelled $PGD_2$-MOX for sites on a specific rabbit antibody. During an incubation period, the rabbit anti- $PGD_2$-MOX antibody becomes bound to a mouse anti-rabbit monoclonal antibody that coats the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution (Ellman's reagent) is added to the wells to determine the bound enzyme activity. The intensity of the colour generated is inversely proportional to the concentration of $PGD_2$ present in the sample (Figure 8).

### 3.5.2 Assay Procedure

A methyl oximating reagent was prepared by diluting supplied methyloxamine HCl in 10 ml of 10% (v/v) solution of ethanol in water. To this was added the supplied sodium acetate to give the final reagent. The $PGD_2$ standard was diluted in 900 µl of MilliQ water and then 1:1 solution was prepared with the methyl oximating reagent. This was heated to 60°C for 30 min resulting in a methoximated standard solution with a concentration of 20 ng/ml. This was used to generate a standard curve by dilution in EIA buffer (7.8 – 1000 pg/ml). Next the cell culture supernatant samples were thawed and 100 µl was added to the same volume of methyl oximating reagent. This solution was heated to 60°C for 30 mins, then centrifuged at 12000g for 5 mins and the supernatant diluted to 1:5 in EIA buffer. The microplate was washed in wash buffer prior to use. 50 µl of standard or sample was added to the microplate coated with mouse anti-rabbit monoclonal antibody. To each was added 50 µl of $PGD_2$ anti-rabbit antibody solution and 50 µl of $PGD_2$ conjugate. The plated were covered and incubated overnight at room temperature. After incubation the wells were aspirated and washed five times with 200 µl wash buffer using a multi-channel pipette, the plate was then inverted and blotted on clean paper towelling to remove all liquid from the wells. 200 µl of Ellman's reagent was added to all wells and incubated for 1 h in the dark at room temperature. The optical density 405 nm was determined using a Dynatech MR5000

platereader. The $PGD_2$ concentrations were calculated by comparison to the standard curve (linear when plotted on log/lin scale) using BioLinx software.

**Figure 8 Schematic representation of prostaglandin ELISAs.** Competitive binding technique used in $PGE_2$ and $PGD_2$ ELISAs



## 3.6 IL-8 ELISA

### 3.6.1 Overview

This assay to measure IL-8 concentration is based on the competitive binding technique in which IL-8 present in the supernatant competes with a fixed amount of labelled IL-8 for sites on a mouse monoclonal antibody. During an incubation period, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody that coats the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The intensity of the colour generated is inversely proportional to the concentration of IL-8 in the sample.

### 3.6.2 Assay procedure

An appropriate number of 96 well microplates (Nunc) were coated with 50 µl per well of monoclonal anti-human IL-8 antibody (2µg/ml, R&D Systems) in PBS pH 7.4. Plates were covered, incubated overnight at room temperature and then washed three times in wash buffer using a multi-channel pipette. Following washing plates were inverted on clean paper towelling to extract any liquid present. The plates were blocked by the addition of 100 µl per well of blocking buffer and left for 1 h at room temperature. Three more washes followed. A range of IL-8 standards were prepared (0.025-2ng/ml) in MEM medium. Standards and samples (50 µl per well) were added in duplicate to the plates and incubated for 2 hours at room temperature. Plates were washed 3 times and blotted and 100 µl per well of biotinylated anti-human IL-8 detecting antibody (20 ng/ml, R&D Systems) in dilution buffer was added to the plates for 1 hour at 37°C. Three more washes and blotting followed and 50 µl per well of streptavidin peroxidase (0.5 µg/ml, R&D Systems) in dilution buffer was added to all wells for 30 min and incubated at 37°C. Plates were washed three times. One 10 mg tablet of 1,2 phenylene dihydrochloride (Sigma) and 20 µl of 30% hydrogen peroxide were added to 50 ml of warmed substrate buffer. This solution was added to the plates (100 µl per well), which were incubated for 30 minutes at room temperature in the dark. The reaction was terminated with 150 µl per well 1 M $H_2SO_4$. The optical density 490 nm was determined using a Dynatech MR5000 plate reader. IL-8 concentrations were

calculated by comparison to the standard curve (linear when plotted on log/lin scale) using BioLinx software.

## 3.7 Glutathione assay

### 3.7.1 Reduced glutathione (GSH) assay

Cells were washed twice and and resuspended in PBS at $1x10^6$ $ml^{-1}$. 500µl aliquots of cell samples were sonicated (3 five second pulses, 8 – 10Hz) to disrupt the cell membranes. 500µl of assay cocktail (40 µM monochlorobimane, 0.1 $u/ml^{-1}$ GSH-S-transferase) was immediately added to both sonicated samples and GSH standards (0, 2, 4, 8, 16, 32, and 64µM), and the samples left to develop fluorescent adduct for ten minutes at room temperature, protected from light. Samples were then measured on a fluorimeter, with a 1cm light path, and excitation wavelength 395nM, emission wavelength 470nM. A standard curve was constructed, and GSH levels in cell samples determined (per 1 x $10^6$ cells).

## 3.8 MTT assay

Cells were grown to 80% confluency, in 3cm plates, and incubated at 37°C in serum free medium. Cells were then either stimulated with either vehicle or ligands as each experiment required. After treatment, monolayers were washed twice with PBS and 500µl of 0.5mg/ml MTT reagent in serum free medium was added to each plate. Plates were incubated for 2 hours at 37°C. After incubation the MTT/serum free medium was aspirated from the plates and 1ml of DMSO added. Plates were swirled for 2 – 3 minutes on a shaker and 100 µl was pipetted off each plate into a 96 well plate, this was performed in triplicate. The 96 well plate was read at 570nm using DMSO as a blank control.

## 3.9 Transfection of cell lines with GFP-linked PKB constructs

Cells were grown, until 40 – 60% confluent, on coverslips, in six-well tissue culture plates at 37°C in a $CO_2$ humidified incubator. Subsequently, 1 μg of DNA and 6μl of PLUS reagent were diluted into 100 μl serum free medium in a sterile tube. In separate tubes lipofectAMINE PLUS reagent diluted in 100 μl serum free medium. These tubes were then combined, mixed gently and incubated at room temperature for 15 minutes. Cells were washed once with serum free medium and replaced with 800 μl of fresh serum free medium. For each transfection the DNA/PLUS reagent and LipofectAMINE PLUS reagent was added to the 800 μl of serum free medium over-laying the cells. Cells were then incubated in the presence of the transfection reagents for three hours at 37°C in a $CO_2$ humidified incubator. After incubation, the DNA-containing medium was replaced with MEM supplemented with 10% foetal calf serum and incubate at 37°C in a $CO_2$ humidified incubator for a further 24 h. Cell extracts were examined for GFP expression using a confocal microscope 24 h post transfection.

## 3.10 Statistical analysis

Duplicate determinations were performed in each experiment. The number (n) of independent experiments is given in the figure legend. Data were analysed by analysis of variance (ANOVA) to determine if any statistical significance existed within data groups. ANOVA was followed by Dunnett's test for the comparison of multiple groups to controls. Data were expressed as means ± SEM of n independent experiments. A probability value of $p<0.05$ was taken as the criterion for significant difference.

# Chapter 4: IL-8 and prostaglandin production in human tracheal epithelial cell lines.

## 4.1 Introduction

Increased levels of PGs have been reported in the sputum of CF patients when compared to healthy individuals (Zakrezewski *et al,* 1987; Strandvik *et al,* 1996). The aim of this work was to examine the production of $PGE_2$ in CF and non-CF human tracheal epithelial cells, using the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines as models of the human airway epithelium. The 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines are both human tracheal epithelial cells exhibiting non-CF and a CF phenotype respectively. In order to determine that these cell lines express functional receptors for pro-inflammatory cytokines and that cytokine signalling pathways are intact, IL-8 levels were measured in each cell line both under basal and stimulated conditions.

## 4.2 Results

### 4.2.1 IL-8 production by human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$ cells

Initial investigations were conducted in order to determine that the cell lines used were responsive to pro-inflammatory cytokines. IL-8 production was investigated using an IL-8 enzyme-linked immunosorbant assay (ELISA), as described in the materials and methods section, under both basal and stimulated conditions. IL-8 levels were determined in cell culture supernatants collected from confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells grown at 37°C and serum starved for 6 hours prior to stimulation with vehicle or increasing concentrations of TNFα (0.01 – 10 ng/ml), IL-1β (0.01 – 10 ng/ml), and IFNγ (0.01 – 10 units/ml) either alone or in combination for a period of 24 hours.

Both 9HTEo$^-$ and ΣCFTE29o$^-$ cells produced a small amount of IL-8 in the absence of cytokine stimulation (1.96 ± 0.40 ng /$10^6$ cells in the 9HTEo$^-$ cell line and 2.24 ± 0.62 ng/$10^6$ cells) in the ΣCFTE29o$^-$ cell line (figure 9). The difference between these levels

was not found to be significant ($n$ = 4, p=0.15). Incubation of the cell lines with either TNFα (figure 9) or IL-1β (figure 10) resulted in a concentration dependent increase in IL-8 levels in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Incubation with TNFα 0.01 and 0.1 ng/ml had no significant effect upon basal IL-8 production in either 9HTEo$^-$ cells or ΣCFTE29o$^-$ cells. In comparison, stimulation of the cells with TNFα (1 and 10 ng/ml) resulted in a significant increase in IL-8 generation in both cell lines when compared to basal ($n$ = 4, p<0.01). It is worth noting that upon incubation with TNFα (10 ng/ml) ΣCFTE29o$^-$ cells produce significantly more IL-8 than 9HTEo$^-$ cells (p<0.05). Incubation of 9HTEo$^-$ cells with IL-1β (0.01ng/ml) induced a significant increase in IL-8 production from basal levels of ($n$ = 4, p<0.05). Incubation with IL-1β (0.1, 1, and 10 ng/ml) further increased IL-8 production in the 9HTEo$^-$ cell ($n$ = 4, p<0.01). In the ΣCFTE29o$^-$ cell line IL-8 production in response to IL-1β (0.01-10ng/ml) exhibited bell-shaped characteristics, IL-1β (0.1 and 1 ng/ml) inducing a significant increase in IL-8 production (n= 4, p<0.01). Additionally, 9HTEo$^-$ cells stimulated with higher concentrations of IL-1β (1 and 10 ng/ml) produced significantly more IL-8 than the ΣCFTE29o$^-$ cell line. Increasing concentrations of pro-inflammatory cytokine IFNγ had no effect on basal IL-8 levels in either cell line (figure 11).

Stimulation of the 9HTEo$^-$ and ΣCFTE29o$^-$ cells with a combination of IFN-γ (1 unit/ml) with IL-1β (0.01 ng/ml) or TNFα (0.1 ng/ml) did not significantly induce IL-8 production in either cell line (figure 12). In combination TNFα (0.1ng/ml) and IL-1β (0.01ng/ml) significantly increased IL-8 production in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines ($n$ = 4, p<0.05). However, the combination of TNFα (0.1ng/ml) and IL-1β (0.01ng/ml) did not cause a significant increase in IL-8 generation when compared to that induced by either TNFα (0.1ng/ml) or IL-1β (0.01ng/ml) individually. Additionally, when these cell lines were stimulated with a combination of IFN-γ (1 unit/ml), IL-1β (0.01 ng/ml), and TNFα (0.1 ng/ml) IL-8 production by both cell lines was significantly increased above basal, and above the level generated in response to TNFα (0.1ng/ml) or IL-1β (0.01ng/ml) individually ($n$ =4, p<0.01).

Incubation of the cell lines with AA had no significant effect on basal IL-8 production by either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines (figure 13). At the concentrations used in this study, 10 and 100 pg/ml, $PGE_2$ also failed to have a significant effect upon IL-8 generation by the tracheal epithelial cell lines used in this study (figure 14).

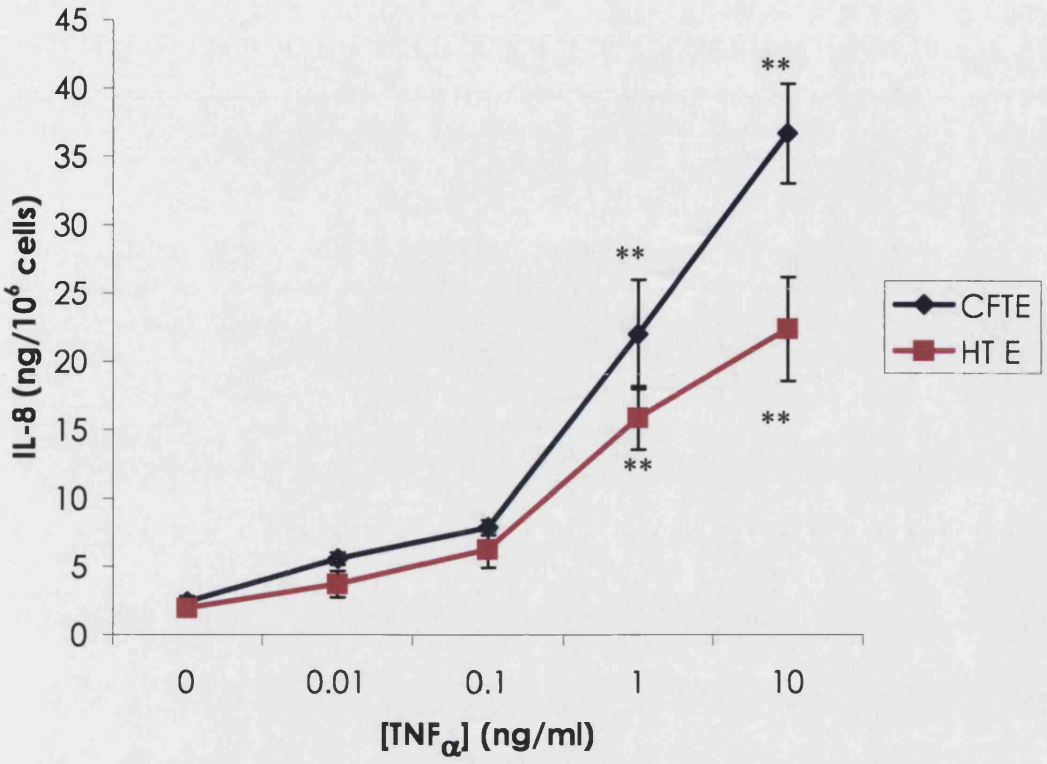



**Figure 9 Effect of TNFα on IL-8 production by 9HTEo⁻ and ΣCFTE29o⁻ cells**

24 h treatment with increasing concentrations of TNFα (0.01 – 10 ng/ml). Cells were serum starved overnight prior to treatment. Basal is the amount of IL-8 produced in response to vehicle alone. Each point is the mean ± SEM of four experiments, samples are run in duplicate in each assay. (**$p<0.01$ compared with basal).

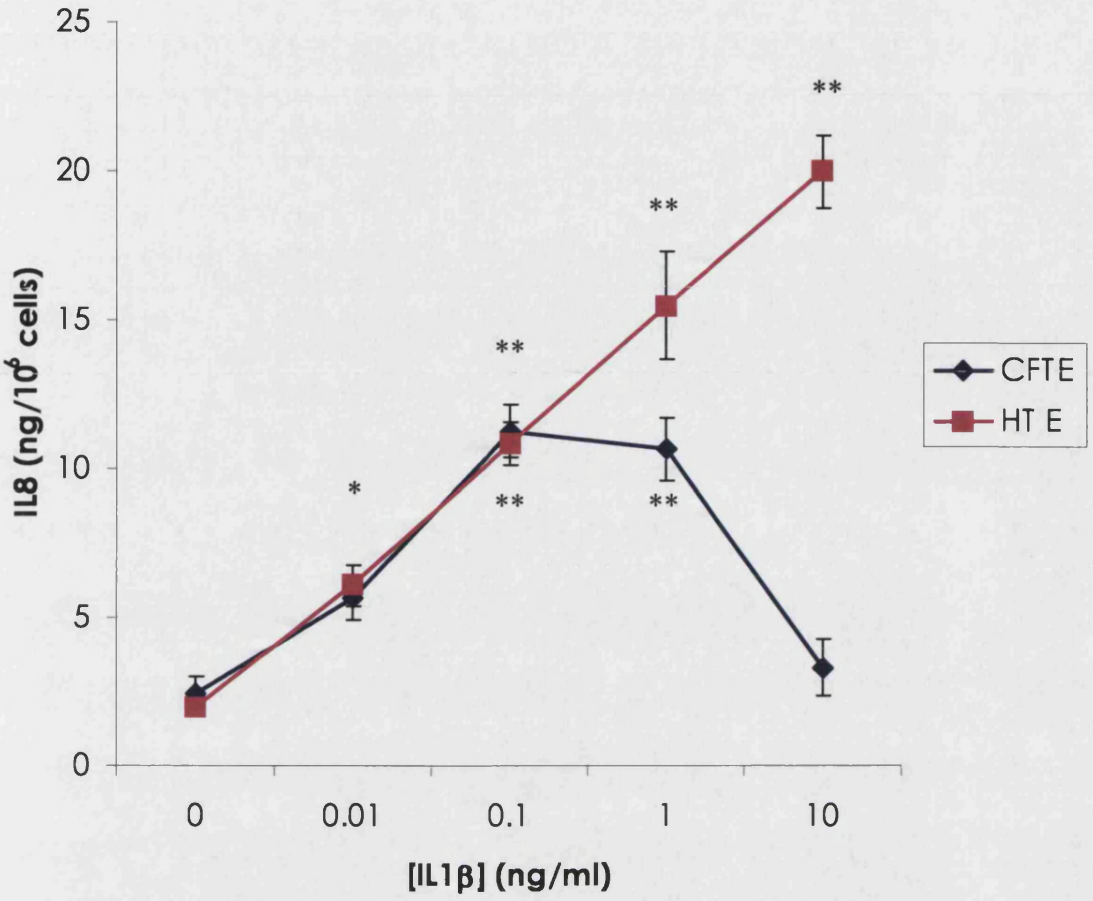



**Figure 10 Effect of IL-1β on IL-8 production by 9HTEo⁻ and ΣCFTE29o⁻ cells**

24 h treatment with increasing concentrations of IL-1β (0.01 – 10 ng/ml). Cells were serum starved overnight prior to treatment. Basal is the amount of IL-8 produced in response to vehicle alone. Each point is the mean ± SEM of four experiments, each sample is measured in duplicate within each assay. (*$p < 0.05$ compared with basal; **$p<0.01$ compared with basal).

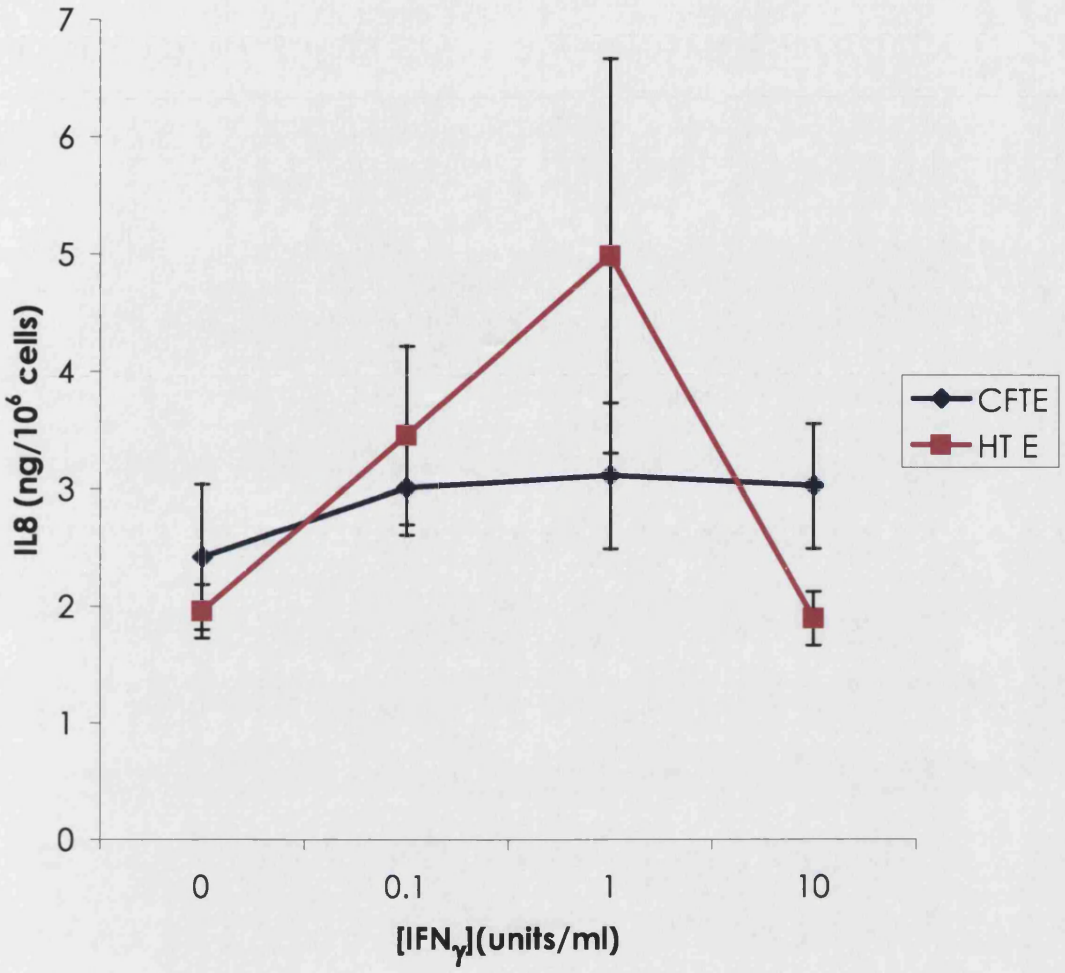



**Figure 11 Effect of IFNγ on IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells**

24 h treatment with increasing concentrations of IFNγ (0 .01 – 10 ng/ml). Cells were serum starved overnight prior to treatment. Basal is the amount of IL-8 produced in response to vehicle alone. Each point is the mean ± SEM of four experiments, each sample is measured in duplicate.

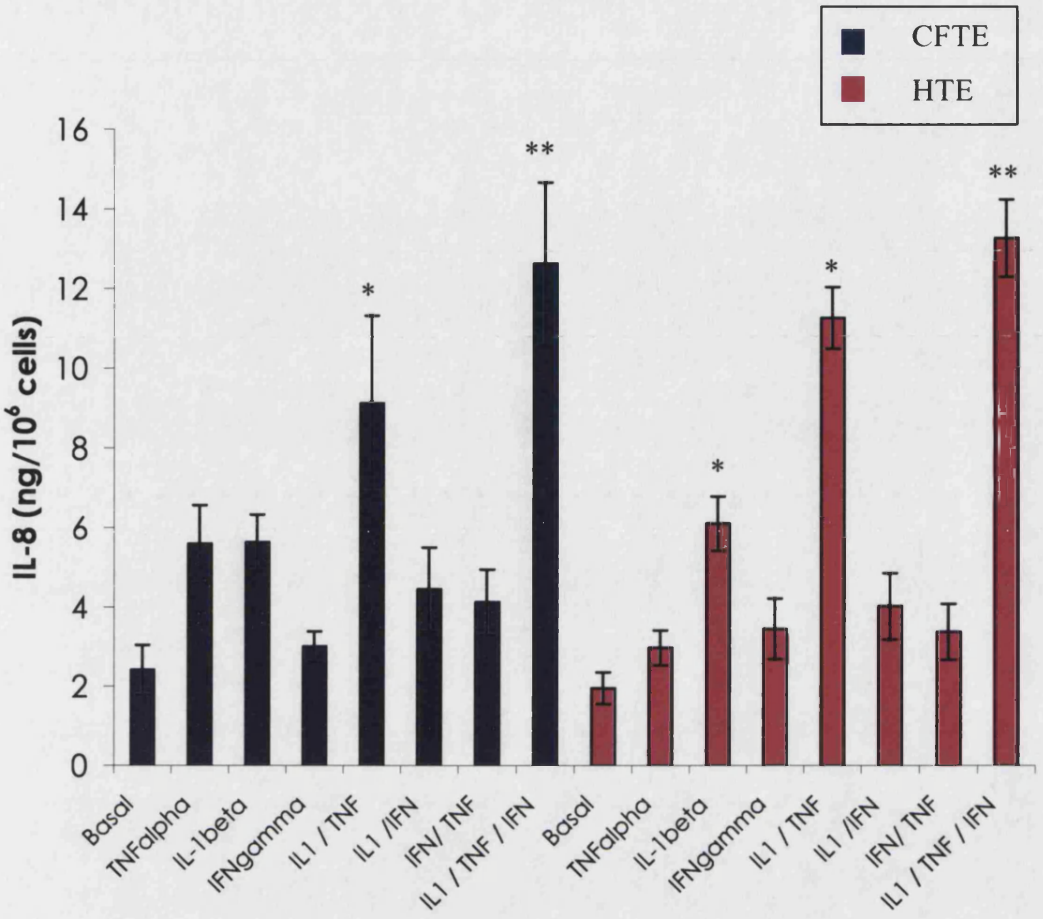



**Figure 12 Effect of cytomix on IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells**

24 h treatment with IFN-γ (1 unit/ml), IL-1β (0.01 ng/ml) or TNFα (0.1 ng/ml) either alone or in combination. Cells were serum starved overnight prior to treatment. Basal is the amount of IL-8 produced in response to vehicle alone. Each point is the mean ± SEM of four experiments each carried out in duplicate. (* $p<0.05$ when compared to basal; ** $p<0.01$ when compared to basal).

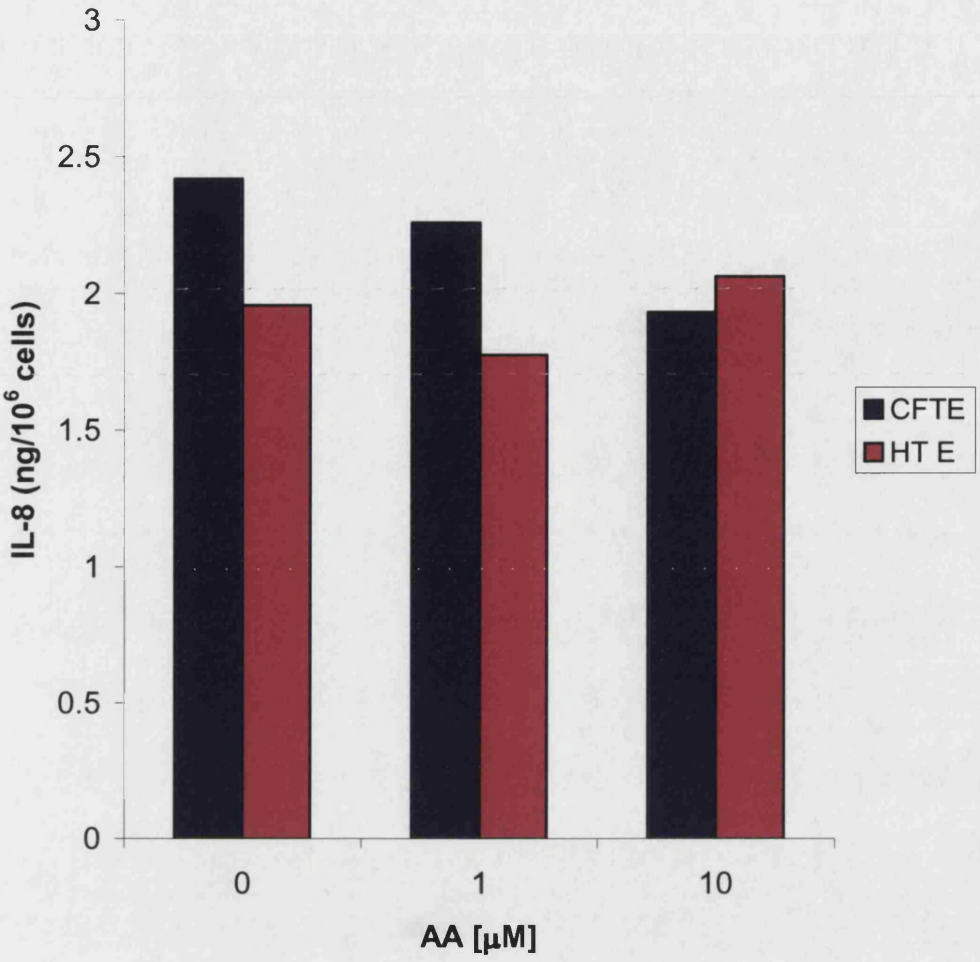



**Figure 13 Effect of AA on IL-8 production by 9HTEo⁻ and ΣCFTE29o⁻ cells**

Effect of 24 h treatment with AA (0 –10 μM). Cells were serum starved overnight prior to treatment. Basal is the amount of IL-8 produced in response to vehicle alone. Each point is the mean of two experiments each carried out in duplicate.

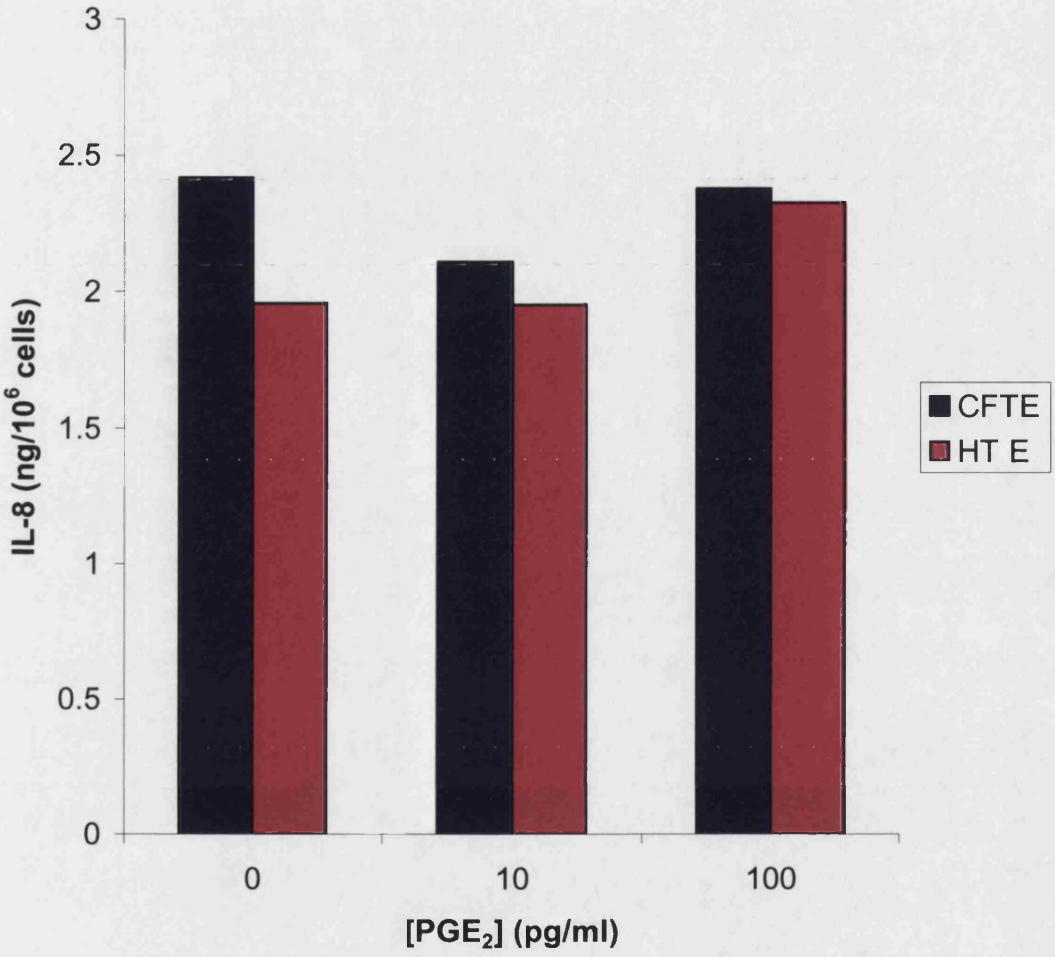



**Figure 14 Effect of $PGE_2$ on IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells**

Effect of 24 h treatment with $PGE_2$ 10 and 100 pg/ml. Cells were serum starved overnight prior to treatment. Basal is the amount of IL-8 produced in response to vehicle alone. Each point is the mean of two experiments each carried out in duplicate.

### 4.2.2 Cyclooxygenase expression in the human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$

Monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were grown, at 37°C, until approximately 90% confluent and subsequently serum starved for 4 hours prior to either harvest or stimulation with pro-inflammatory cytokines. COX-1 and COX-2 protein expression was determined by immunoblot analysis as described in the materials and methods section. Both 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines constitutively express COX-1 protein (figures 15A and 15B), however COX-1 expression in the 9HTEo$^-$ cell line is higher than that of the ΣCFTE29o$^-$ cell line. As expected, COX-1 protein expression was not induced by TNFα (0.1 - 10 ng/ml), IL-1β, (0.1 – 10 ng/ml), (figures 15A and 15B) IFNγ (0.1 – 10 units/ml) or a combination of all three of these pro-inflammatory cytokines (figures 16A and 16 B).

COX-2 protein expression was observed in untreated 9HTEo$^-$ and ΣCFTE29o$^-$ cells with no obvious difference in expression between these two cell lines (figure 17). COX-2 protein expression has been reported to be up-regulated in response to pro-inflammatory cytokines in a number of airway cell lines (Mitchell *et al.,* 1994; Watkins *et al.,* 1999; Rodgers *et al.,* 2002). To investigate the time course of COX-2 protein induction in response to pro-inflammatory cytokines cells were incubated at 37°C with a combination of TNFα (10 ng/ml), IL-1-β (1 ng/ml) and IFNγ (10 u/ml) and subsequently harvested 2, 6, 14, and 24 hours post stimulation, concomitantly untreated control samples were collected for each time point. No up-regulation of COX-2 expression was observed in response to the cytomix compared to control samples at any of the time points (figures 18 A and 18B). Additionally, COX-2 protein expression was not significantly affected by any of these cytokines administered individually at these time points. After 48 hours or when incubated with higher concentrations of cytokines the viability of the cells decreased. Microscopic examination revealed a decrease in cell number, and an increase in cell debris with cell viability typically reducing to >50% in the 9HTEo$^-$ cell line. In view of this, time courses were routinely constructed over a 24 hour period. In order to determine any dose – dependent effect of pro-inflammatory cytokines on COX-2 protein 9HTEo$^-$ and ΣCFTE29o$^-$ cells were incubated at 37°C with TNFα (0.1 - 10 ng/ml), IL-1β (0.1 – 10

ng/ml), IFNγ (0.1 - 10 units/ml) either alone or in combination for 24 hours. Once again no significant increase in COX-2 expression was detectable in either cell line in response to any of the cytokines used in this study (figures 19 A, B, C and D). Additionally, cells were serum starved for 6 hours prior to 24 hour stimulation, at 37°C, with increasing concentrations of IL-13 (0.1 – 10 ng/ml). The cytokine IL-13 has been demonstrated to negatively regulate COX-2 protein expression in lung fibroblasts (Saito *et al.,* 2003), thus the effect of IL-13 on COX-2 expression was investigated in ΣCFTE29o$^-$ and 9HTEo$^-$ cells. No detectable change in COX-2 protein expression was observed in either 9HTEo$^-$ or ΣCFTE29o$^-$ cells in response to any of the concentrations of IL-13 used in this study (figure 20).

PGs are products of COX enzymes, however, it has also been demonstrated that they are able to modulate the expression of COX-2 (Mitchell *et al.,* 1994; Belvisi *et al.,* 1997; Pang *et al.,* 1998; Bonazzi *et al.,* 2000). In order to determine whether $PGE_2$ concentration affects the expression of COX-2 in 9HTEo$^-$ and ΣCFTE29o$^-$ cells, monolayers of these cells were grown, at 37°C, until approximately 90% confluent and subsequently serum starved for 4 hours prior to stimulation with vehicle or increasing concentrations of $PGE_2$ for 24 h. COX-2 protein expression was detected using immunoblot analysis as described in the materials and methods section. No change in COX-2 protein expression could be detected in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell line in response to any of the concentrations of $PGE_2$ used in this study (figures 21A and 21B). Both cell lines were also incubated, for 6 hours, with either vehicle or $PGE_2$ to ensure that no earlier change in COX-2 protein expression had been missed. Once again no detectable change in COX-2 protein was evident (figure 22).

NSAIDs exert their activity by inhibiting the ability of COX enzymes to convert AA into $PGG_2$ and $PGH_2$. However, NSAIDs have also been observed to modulate COX-2 expression in a cell type specific manner (Pang *et al.*, 2003). In order to determine whether NSAIDs have a significant effect upon COX-2 protein expression in 9HTEo$^-$ and ΣCFTE29o$^-$ cells, monolayers of confluent cells were starved for 4 hours and subsequently incubated with either the non-specific COX inhibitor indomethacin (10 μM), COX-2 specific inhibitor NS398 (10 μM), or acetaminophen (10 μM), a reducing agent of both COX-1 and COX-2 (Oullet and Percival, 2001), for 24 hours in the

presence or absence of TNFα 10 (ng/ml). Cells were pre-incubated with the NSAIDs 30 min prior to the addition of TNFα. Samples were collected and COX–2 protein expression determined by immunoblot analysis.

No increase in COX-2 protein expression could be detected in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell line in response to the NSAIDs used either individually or in combination with TNFα nor could any increase in COX-2 protein expression be observed in response to TNFα alone this correlating with earlier observations (figures 23 A, B, and C).

Attempts were made to transfect monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells with a COX-2 promoter construct in order to assess mechanisms involved in the transcriptional regulation of COX-2 in human tracheal epithelial cell lines. Preliminary experiments were carried out to optimise lipofectAMINE plus transfection of these cell lines using a green fluorescent protein (GFP) -linked PKB construct, as described in the materials and methods section. This construct was used as it enabled visualisation of the transfection process (figure 24). Unfortunately, these initial experiments revealed that the 9HTEo$^-$ cell line was unable to survive incubation with lipofectAMINE plus. Furthermore, effective transfection of the ΣCFTE29o$^-$ cell line was not achieved within the timescale of this study.

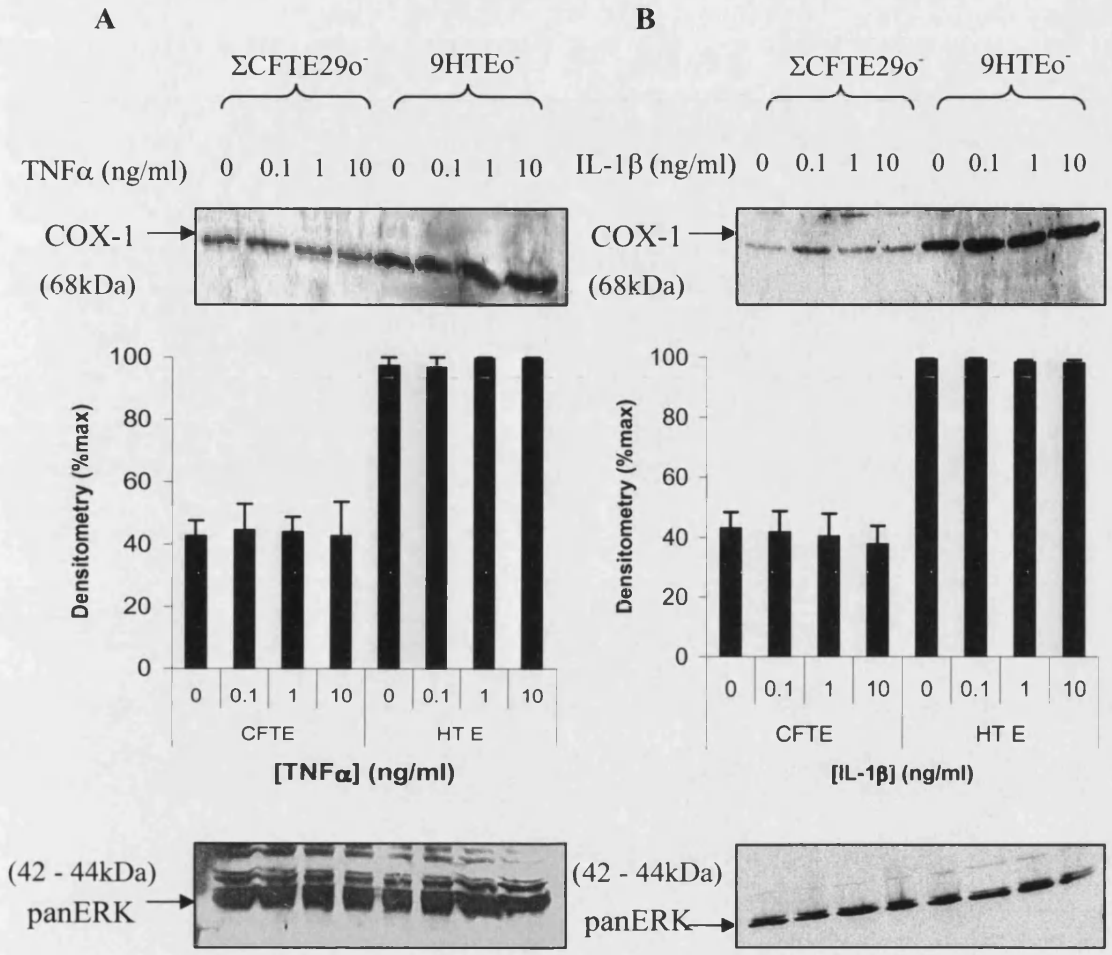



**Figure 15 Effect of cytokines on expression of cyclooxygenase-1 (COX-1) in ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of exogenous application of TNFα (0.1 – 10 ng/ml) over 24 hours. B) Effect of exogenous application of IL-1β (0.1 – 10 ng/ml) over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-1. This immunoblot is representative of at least 2 other experiments. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 3 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

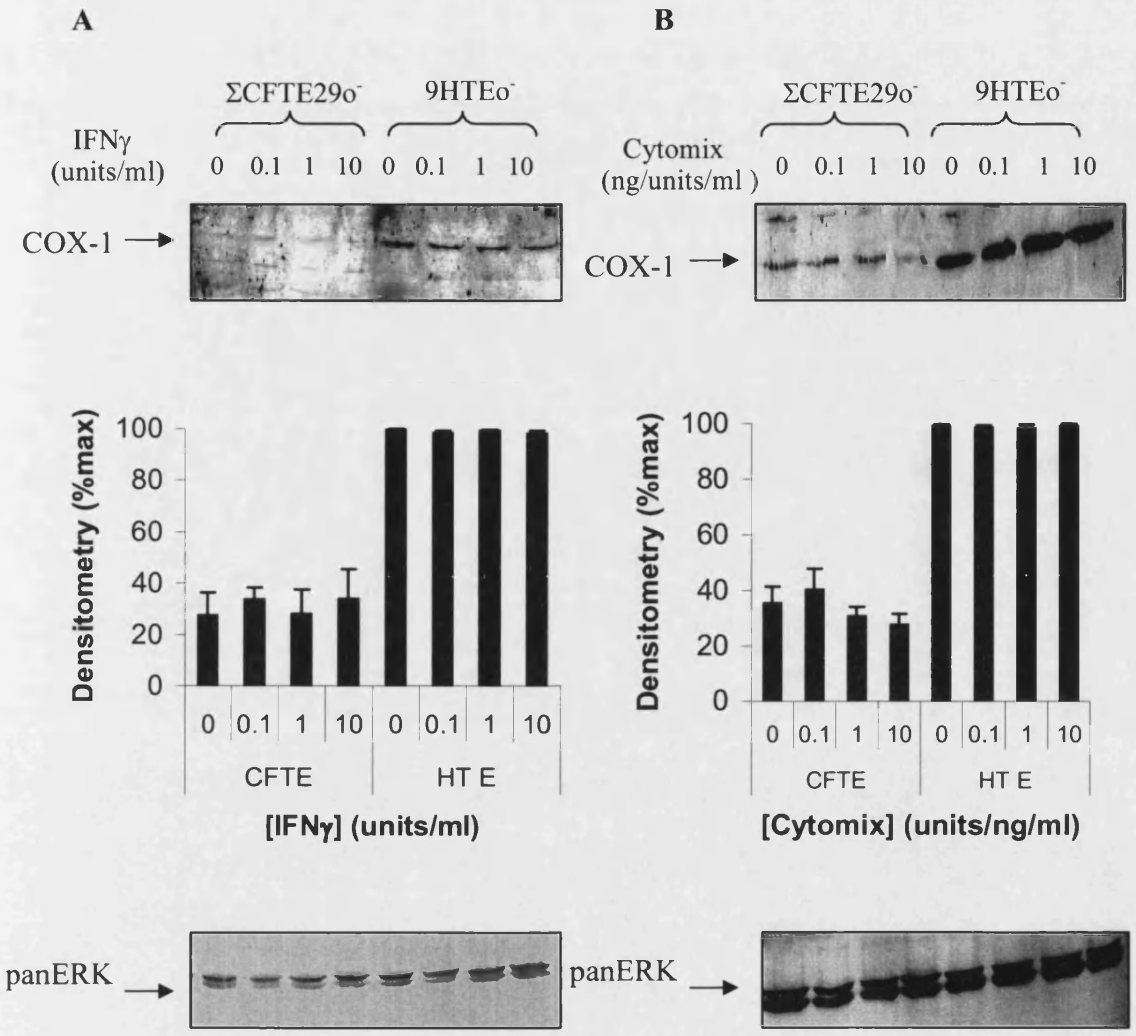



**Figure 16 Effect of cytokines on expression of COX-1 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of IFNγ (0.1 – 10 ng/ml) over 24 hours. B) Effect of exogenous application of a cytomix of IFNγ (0.1 – 10 units/ml), TNFα (0 – 10 ng/ml) and IL-1β (0.1 – 10 ng/ml) over 24 hours.Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-1. This immunoblot is representative of 2 other experiments. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 3 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

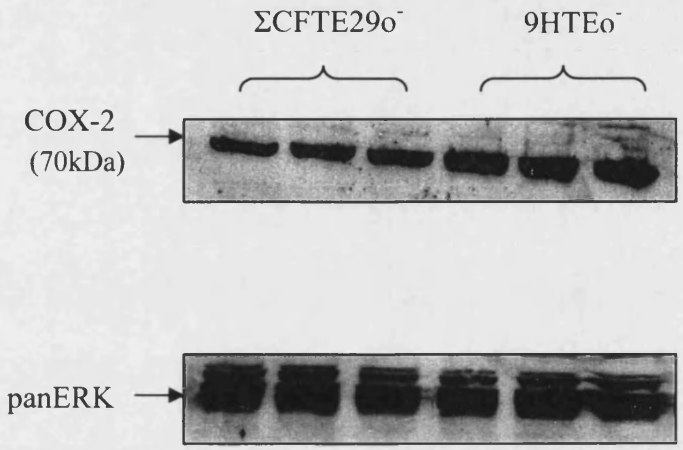



**Figure 17 Basal expression of cyclooxygenase-2 (COX-2) in untreated ΣCFTE29o⁻ and 9HTEo⁻ cells.**

Western blot analysis of whole cell lysates. The top panel is the immunoblot probed with a specific antibody against COX-2. Lanes 1 – 3 are unstimulated ΣCFTE29o⁻ samples from 4 separate experiments. Lanes 4 – 6 are unstimulated 9HTEo⁻ samples from 4 separate experiments. This immunoblot is representative of at least 5 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

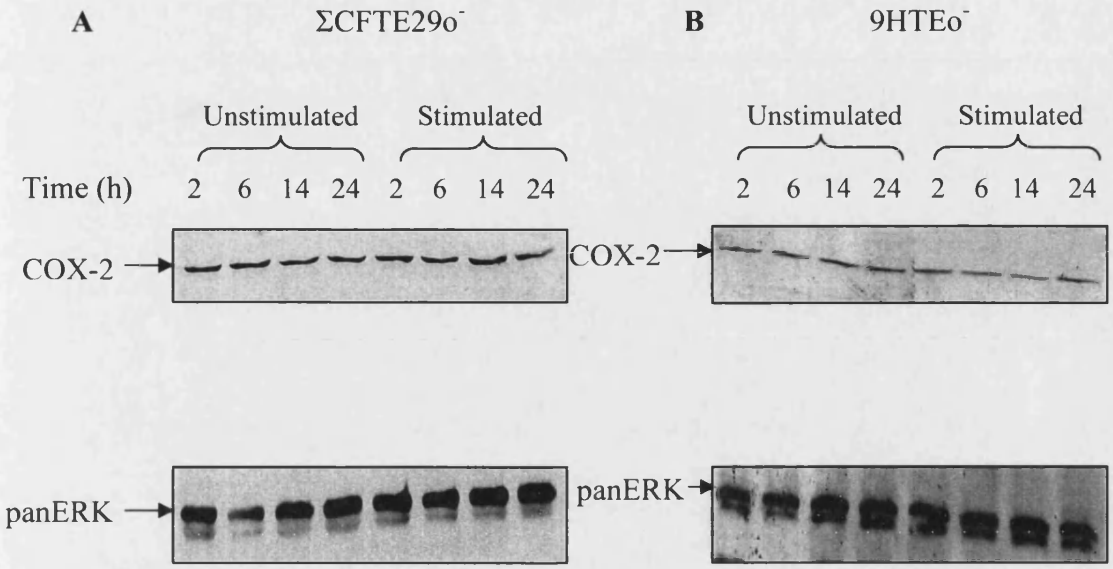



**Figure 18 Time course of effect of cytomix on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of a cytomix of IFNγ (10 units/ml), TNFα (10ng/ml) and IL-1β over a time course of 2, 6, 14, and 24 hours on the ΣCFTE29o⁻ cell line B) Effect of exogenous application of a cytomix of IFNγ (10 ng/ml), TNFα (10ng/ml) and IL-1β over a time course of 2, 6, 14, and 24 hours on the 9HTEo⁻ cell line.Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. These immunoblots are representative of at least 1 other experiment. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

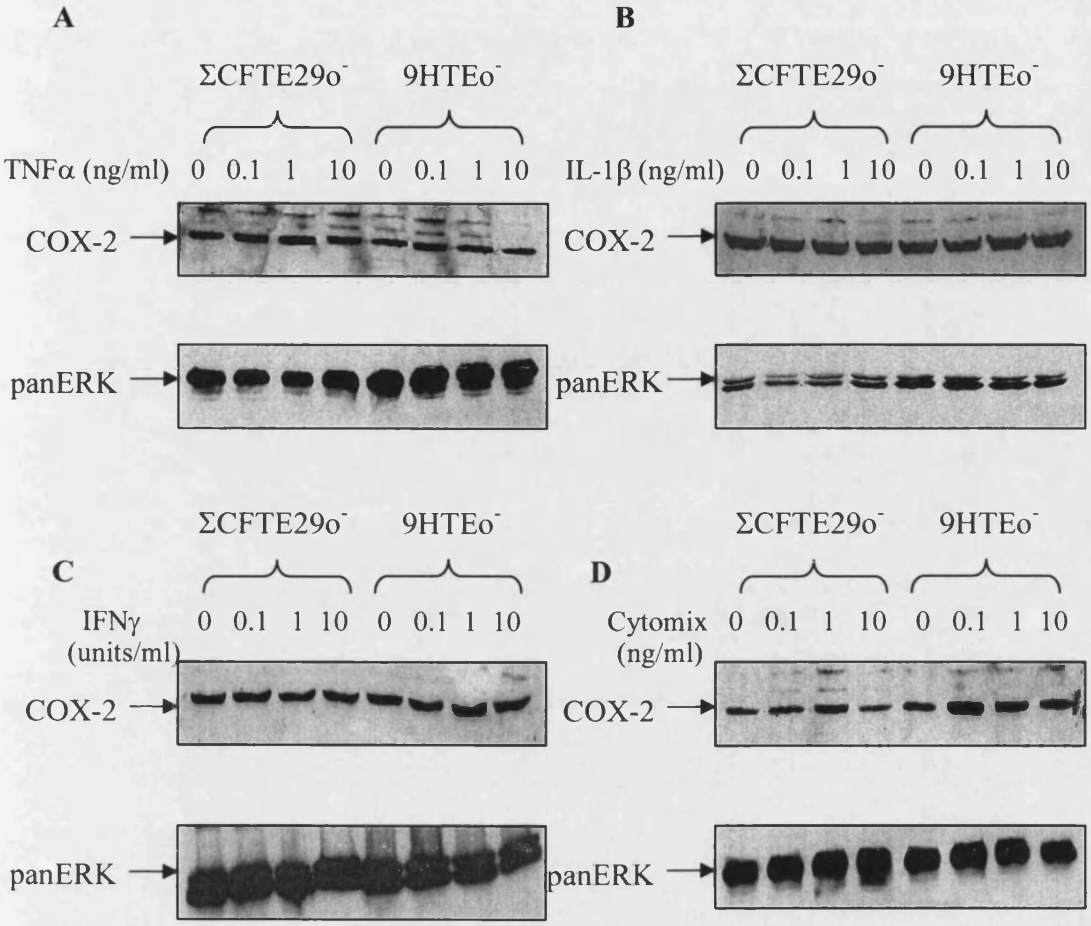



**Figure 19 Effect of cytokines on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of TNFα (0 – 10ng/ml), over 24 hours. B) Effect of exogenous application of increasing concentrations of IL-1β (0 – 10ng/ml), over 24 hours. C) Effect of exogenous application of increasing concentrations of IFNγ (0 – 10units/ml), over 24 hours. D) Effect of exogenous application of a cytomix of IFNγ (0 – 10 units/ml), TNFα (0.1 - 10ng/ml) and IL-1β (0 – 10ng/ml) over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. These immunoblots are representative of at least 3 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

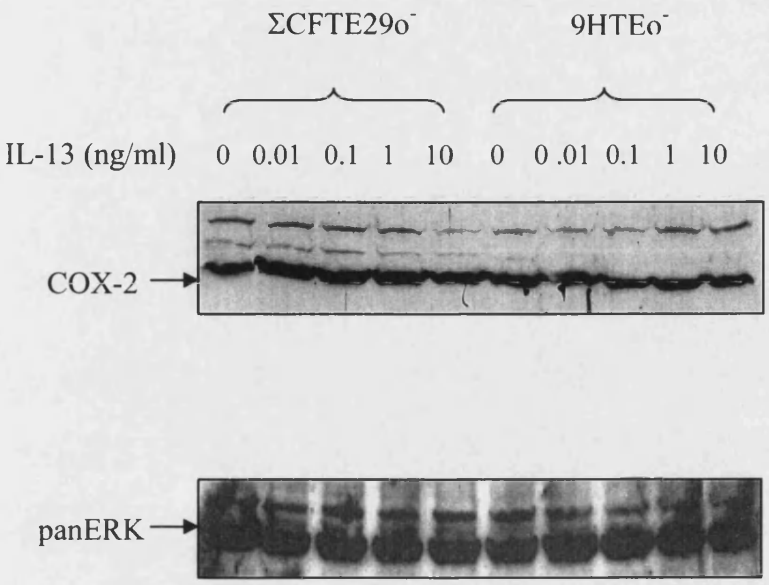



**Figure 20 Effect of IL-13 on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

Effect of exogenous application of increasing concentrations of IL-13 (0 – 10ng/ml), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. This immunoblot is representative of at least 2 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

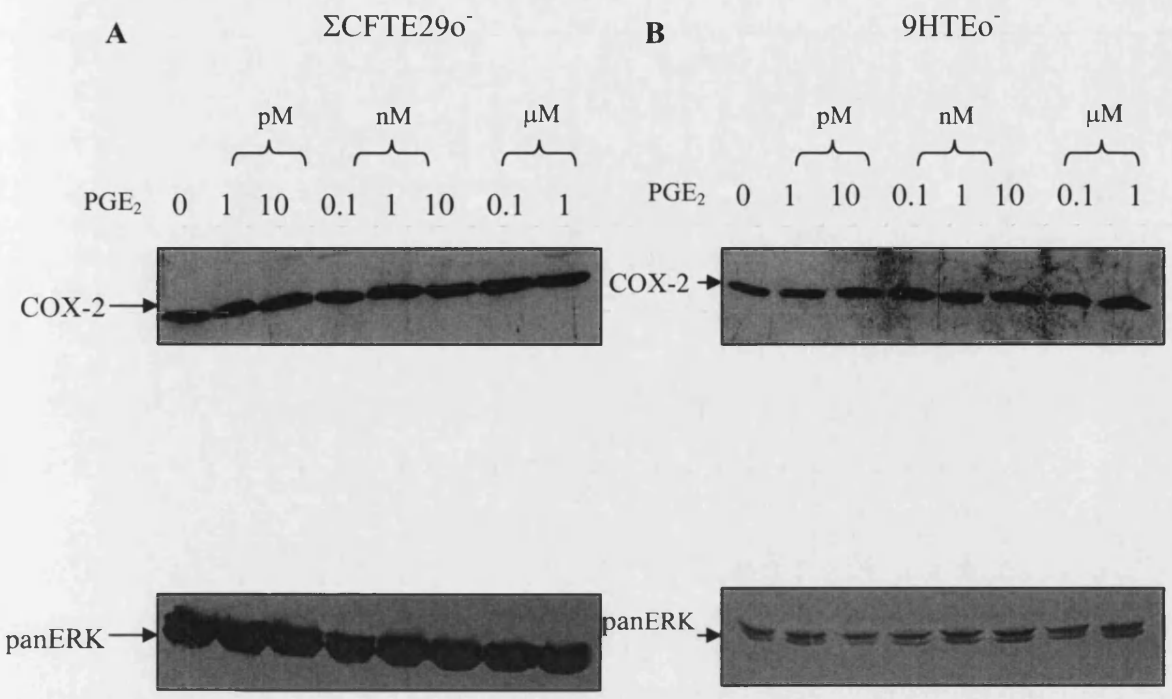



**Figure 21 Effect of $PGE_2$ on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of $PGE_2$ (1pM – 1μM), over 24 hours, on the ΣCFTE29o⁻ cell line. B) Effect of exogenous application of increasing concentrations of $PGE_2$ (1pM – 1μM), over 24 hours on the 9HTEo⁻ cell line. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. These immunoblots are representative of 1 other experiment. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

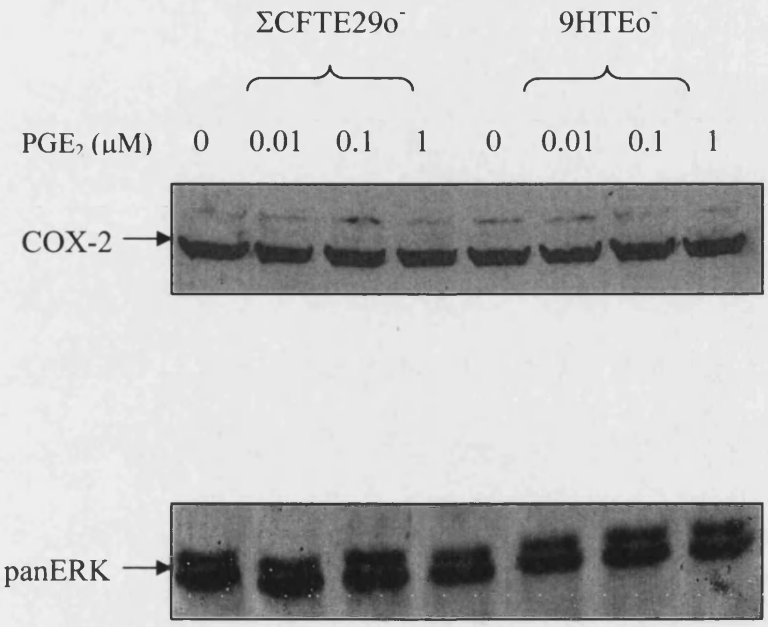



**Figure 22 Effect of $PGE_2$ on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of exogenous application of increasing concentrations of $PGE_2$ (0.01 – 1μM) over 6 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

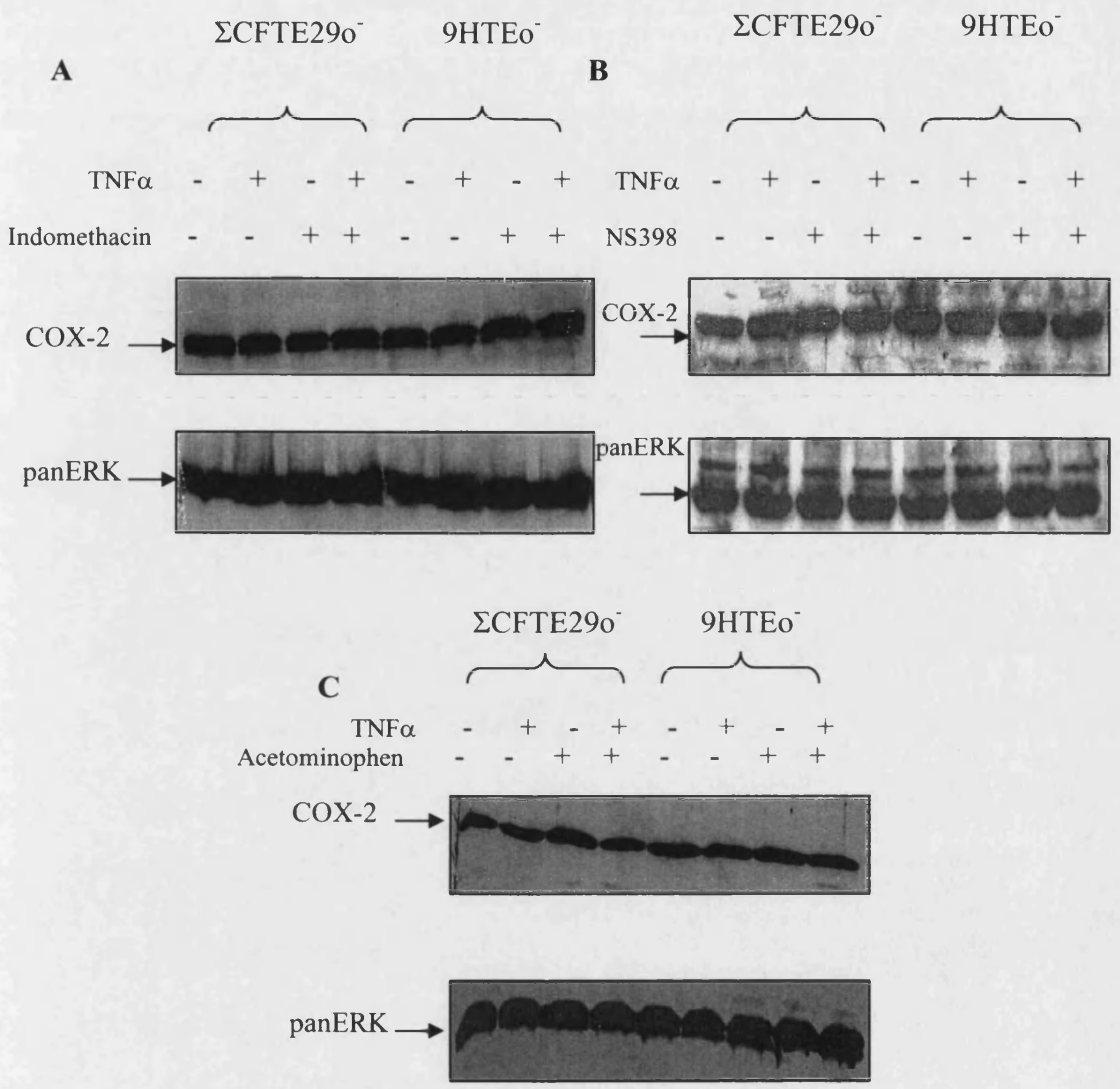



**Figure 23 Effect of NSAIDs on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of TNFα (10ng/ml) with or without indomethacin (10μM), over 24 hours. B) Effect of exogenous application of TNFα (10ng/ml) with or without NS-398 (10μM), over 24 hours. C) Effect of exogenous application of TNFα (10ng/ml) with or without acetaminophen (10μM), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

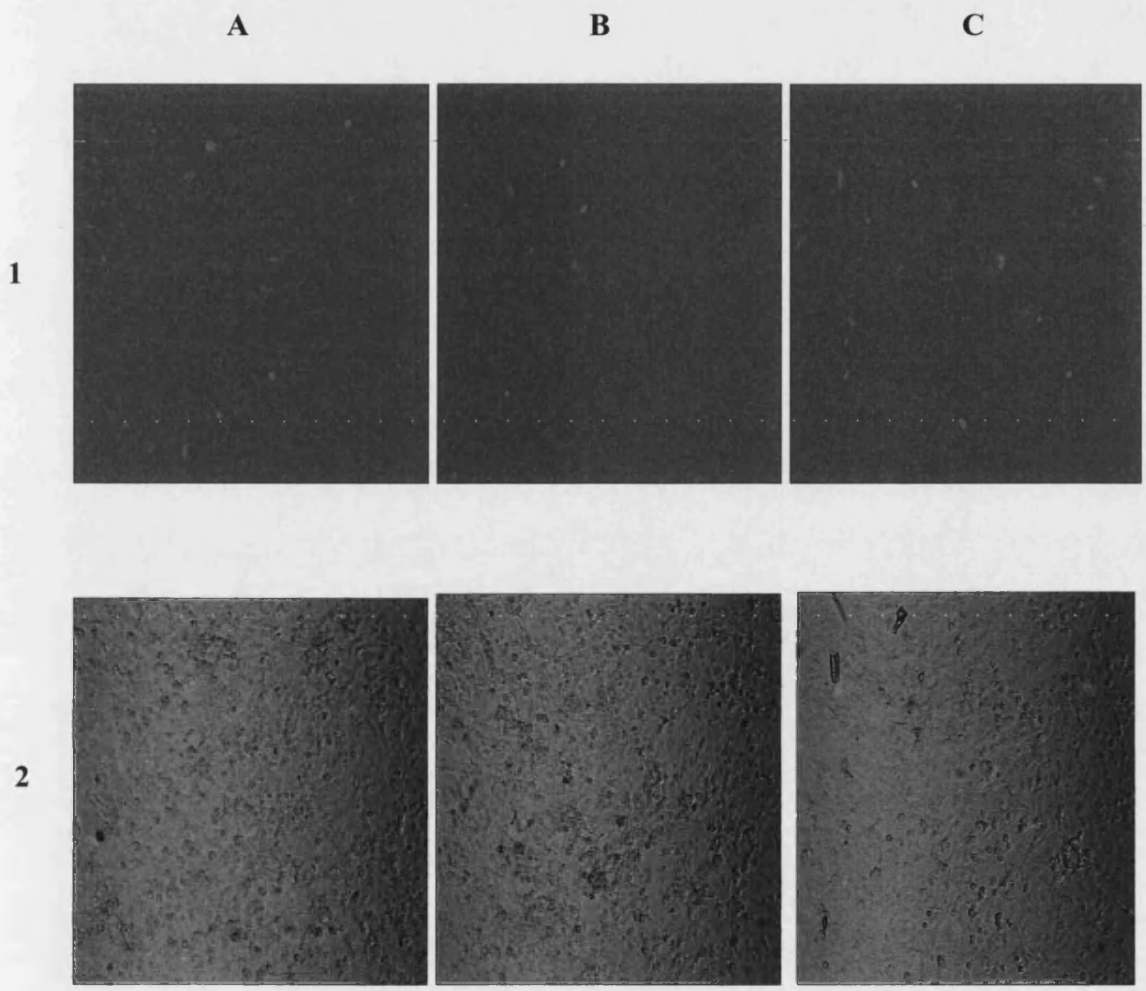



**Figure 24 Transfection of the ΣCFTE29o⁻ cell line with a GFP-tagged PKB promoter construct.**

Monolayers of cells 40 – 60% confluent growth-arrested, and transfected using 1 μg of DNA/well and A) 1 μg lipofectAMINE plus B) 3 μg lipofectAMINE plus and C) 6 μg lipofectAMINE plus. Expression of construct expression was visualised using a confocal microscope at 24 hours post-transfection.

### 4.2.3 Expression of the prostaglandin E synthases in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$

The COX enzymes synthesise $PGH_2$ from AA. $PGH_2$ is subsequently converted into $PGE_2$ by the action of PGE synthase enzymes cPGES, believed to be a constitutive isoform functionally linked to COX-1, and mPGES, an inducible isoform functionally coupled to COX-2. Confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 4 hours and cPGES protein expression was determined by immunoblot analysis. Figure 25 shows the results of a time course study carried out to determine the basal expression of cPGES and to investigate whether TNFα (10 ng/ml) has any effect on cPGES over a 48 hour period. As expected cPGES protein was found to be constitutively expressed in both 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines with no observable change in this isoforms expression in response to TNFα when compared to basal (figures 25 A and B).

The type II alveolar carcinoma cell line A549 was used as a positive control to demonstrate that mPGES expression and up-regulation are detectable under the conditions implemented in this study. Monolayers of A549, 9HTEo$^-$ and ΣCFTE29o$^-$ cells were grown at 37°C until confluent and serum starved for 4 hours prior to a 24 hour incubation with vehicle or TNFα (10ng/ml). The A549 cell line constitutively expresses mPGES protein, incubation of these cells for 24 hours increases mPGES expression from approximately 45% of maximum to 100% (figure 26). No mPGES protein was detectable in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines in the presence or absence of TNFα (figure 26).

To investigate whether $PGE_2$ exerts an effect upon either cPGES or mPGES expression, confluent monolayers of A549, 9HTEo$^-$ and ΣCFTE29o$^-$ cells were grown at 37°C until confluent and serum starved for 4 hours prior to a 24 hour incubation with vehicle or $PGE_2$ (0.1 – 10 nM). No significant change in either cPGES (figure 27 A) or mPGES (figure 27 B) protein expression, when compared to basal, could be observed in 9HTEo$^-$ or ΣCFTE29o$^-$ cells in response to $PGE_2$.

Both cPGES and mPGES are glutathione (GSH) dependent enzymes, requiring GSH for optimal activity. Basal glutathione levels in 9HTEo$^-$ and ΣCFTE29o$^-$ cells were determined using a glutathione assay, as described in the materials and methods section. Confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were starved for 4 hours prior to resuspension in PBS and sonication to disrupt the cell membranes. No significant difference was found between basal glutathione levels in the 9HTEo$^-$ cell line 2.5 ± 0.24 μM/$10^6$ and the ΣCFTE29o$^-$ 3.10 ± 0.39 μM/$10^6$ ( $n$ = 5, p=0.250) (figure 28).

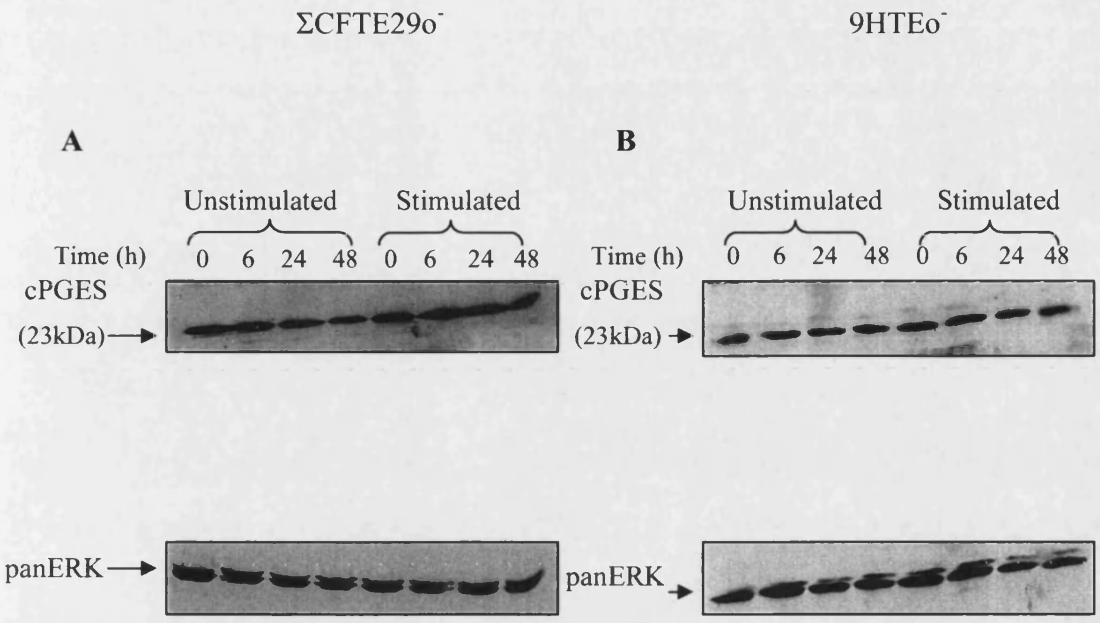



**Figure 25 Effect of TNFα on expression of cPGES protein in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Time course of cPGES expression in ΣCFTE29o⁻ cells after incubation with vehicle or TNFα (10ng/ml) over a 48 hour period B) Time course of cPGES expression in 9HTEo⁻ cells after incubation with vehicle or TNFα (10ng/ml) over a 48 hour period. Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against cPGES. These immunoblots are representative of at least 2 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

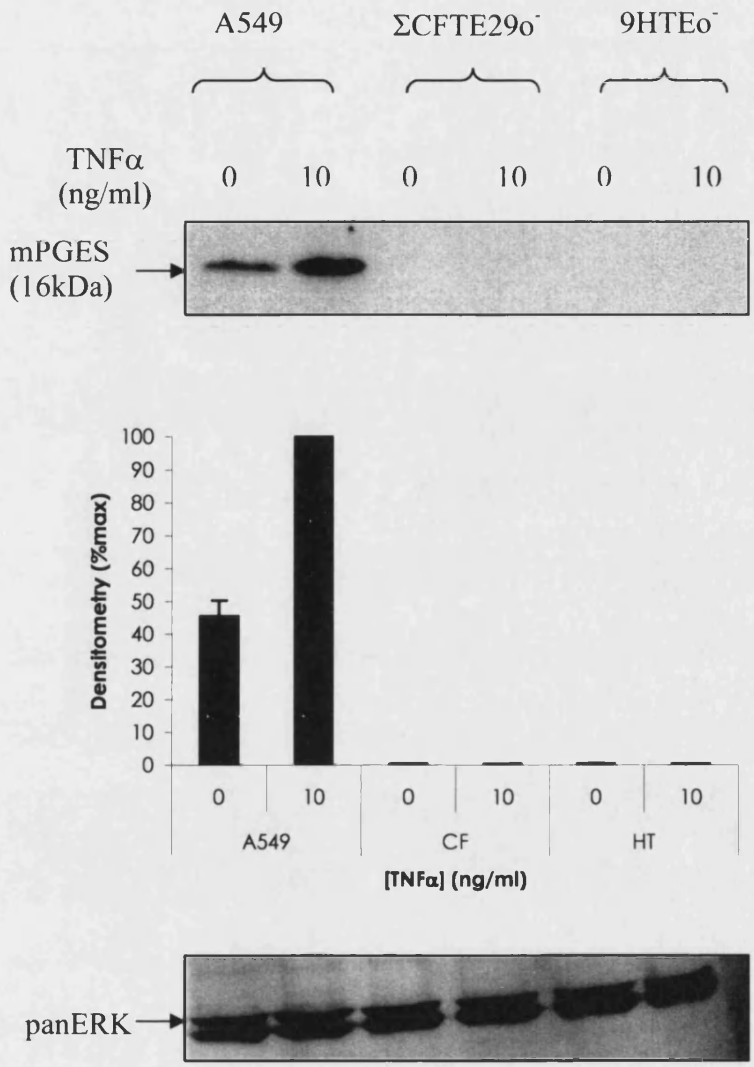



**Figure 26 Effect of TNFα on expression of mPGES protein in A549, ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

Effect of exogenous application of increasing concentrations of TNFα (0.1 - 10 ng/ml), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against mPGES, this immunoblot is representative of three others. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 4 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

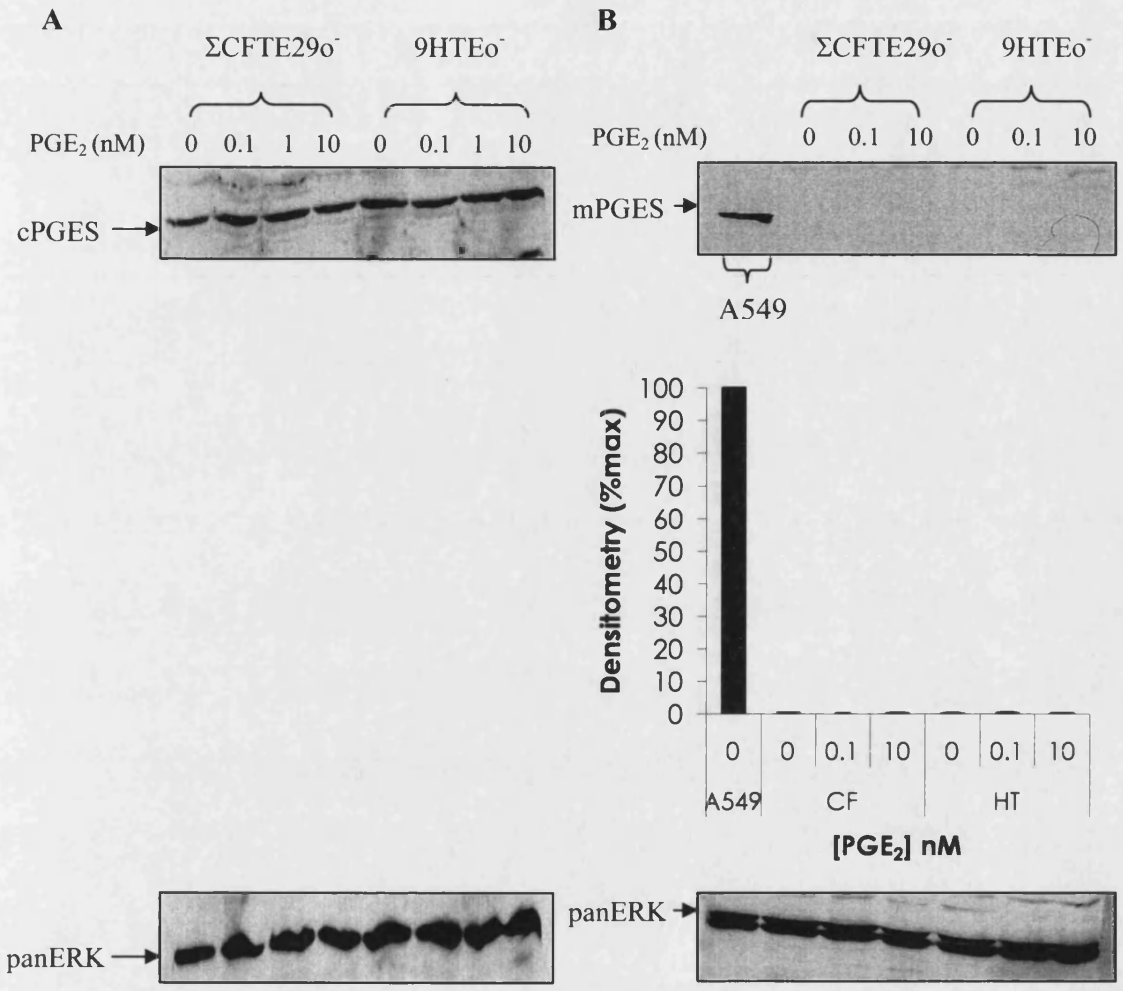



**Figure 27 Effect of $PGE_2$ on expression of cPGES and mPGES proteins in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of $PGE_2$ (0.1 - 10 nM), over 24 hours, on cPGESprotein expression B) Effect of exogenous application of increasing concentrations of $PGE_2$ (0.1 - 10 nM), over 24 hours on mPGES protein expression in A549, ΣCFTE29o⁻ and 9HTEo⁻ cell lines. Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against A) cPGES and B) mPGES, these immunoblots are each representative of three others. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 4 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

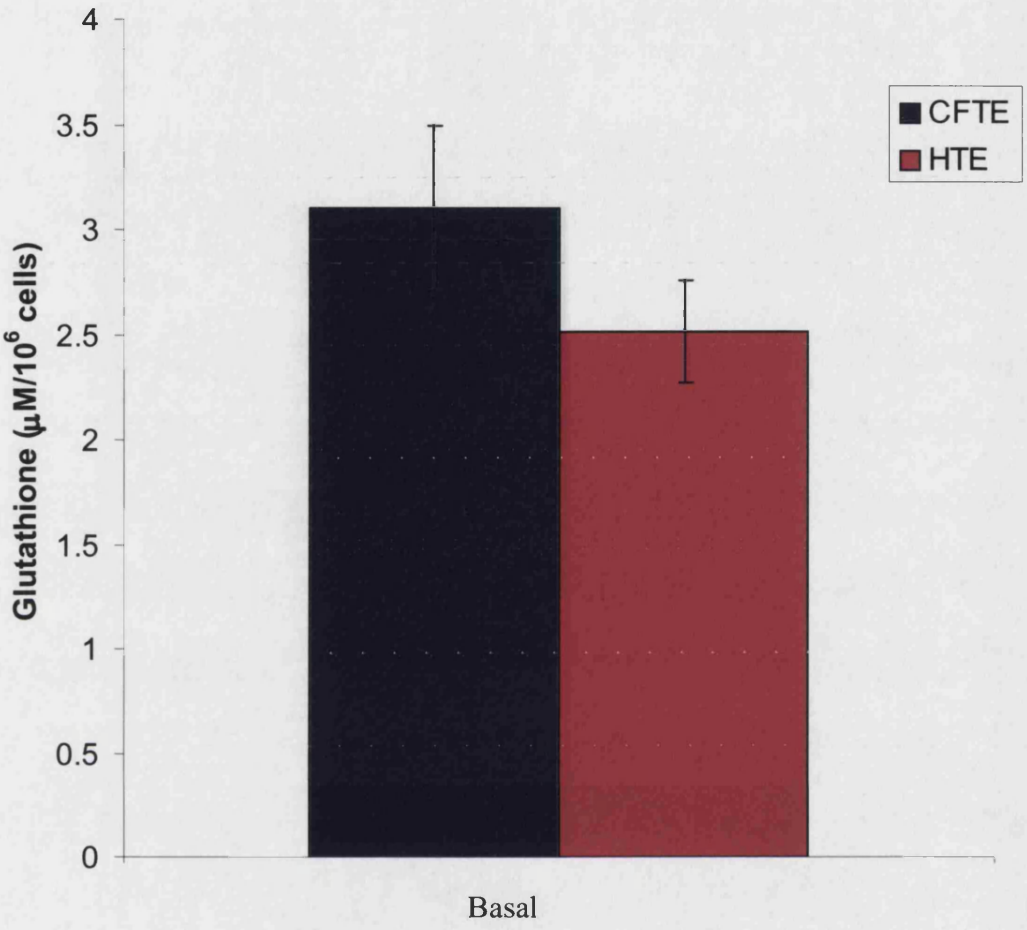



**Figure 28 Basal glutathione levels in ΣCFTE29o⁻ and 9HTEo⁻ cells**

The cells were serum starved for 4 hours prior to incubation with a cocktail of 40 μM monochlorobimane and 0.1 units/ml$^{-1}$ GSH-S-transferase and subsequent analysis. Each bar is the mean ± SEM of 5 experiments carried out in duplicate.

### 4.2.4 Prostaglandin production by the human tracheal epithelium

$PGE_2$ is the most common PG produced in the airways and is thought to be predominantly a COX-2 product. In order to investigate the production of $PGE_2$ by ΣCFTE29o$^-$ and 9HTEo$^-$ cells, monolayers of cells were grown until confluent and serum starved for 6 hours prior to incubation, at 37°C, with either vehicle, cytokines, or AA. Subsequently, $PGE_2$ levels were determined in cell culture supernatants using a $PGE_2$ ELISA as described in the materials and methods section.

Initial studies were carried out in 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cells, $PGE_2$ was produced under basal conditions by both cell lines. However, basal levels of $PGE_2$ observed in the ΣCFTE29o$^-$(1) cell line were approximately ten-fold higher than those of the 9HTEo$^-$ cell line (figure 29). Stimulation of ΣCFTE29o$^-$(1) cells with pro-inflammatory cytokines IL-1β, TNFα and IFNγ individually failed to induce $PGE_2$ production, however a cytomix of all three cytokines induced a significant increase in $PGE_2$ generation by this cell line (figure 29). In contrast, $PGE_2$ production by the 9HTEo$^-$(1) could not be induced by any of these cytokines either alone or in combination (figure 29). Problems with the growth of these cells resulted in a new batch of 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cells being ordered from the original source. These new cells, the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines were used for the rest of this study. Furthermore, the data already shown in this report was obtained from the new batch of cells. The data obtained from these new cells differs in some aspects from those seen with the original 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines. As the new batch have been handled by the same person from arrival and their growth and data obtained consistent, the data from the 9HTEo$^-$ and ΣCFTE29o$^-$ cells are taken to be accurate. Therefore, the 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines were put aside and the 9HTEo$^-$ and ΣCFTE29o$^-$ cells used throughout this study.

Both 9HTEo$^-$ and ΣCFTE29o$^-$ cells produced $PGE_2$ in the absence of stimulation. However, basal levels of $PGE_2$ observed in the ΣCFTE29o$^-$ cell line were approximately seven-fold higher than those of the 9HTEo$^-$ cell line ($n$ = 5, $p<0.01$) (figure 30). The release of $PGE_2$ by the 9HTEo$^-$ cell line was elevated over basal levels when incubated for 24 hours with TNFα (10 ng/ml) ( $n$ = 3, $p<0.05$) and TNFα in

combination with IL-1β (both 10ng/ml) ( $n$ = 3, $p<0.05$). IL-1β (10 ng/ml) and IFNγ (10 units/ml) had no significant effect on $PGE_2$ production, by the 9HTEo$^-$ cell line, either alone or in combination. The greatest increase in $PGE_2$ generation observed at the 24 hour time point, in this cell line, occurred after stimulation with a combination of IL-1β (10 ng/ml), TNFα (10 ng/ml), and IFNγ (10 units/ml) ($n$ = 3, $p<0.01$). In the ΣCFTE29o$^-$ cell line only the cytomix of IL-1β (10 ng/ml), TNFα (10 ng/ml), and IFNγ (10 units/ml) induced a significant increase, from basal levels, of $PGE_2$ generation at the 24 hour time point ($n$ = 3, $p<0.01$) (figure 31). These data are similar to that gathered from the 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines (figure 29), however differences are seen in the inducibility of $PGE_2$ production by pro-inflammatory cytokines.

Time course studies were carried out in order to investigate if stimulation of the airway epithelial cells with TNFα 10 ng/ml or the cytomix of IL-1β (10 ng/ml), TNFα (10 ng/ml), and IFNγ (10 units/ml) resulted in induction of $PGE_2$ at time points other than 24 hours. These studies revealed that incubation of 9HTEo$^-$ cells with TNFα resulted in a significant increase in $PGE_2$ generation at the 2 hour time point ($n$ = 3 $p<0.05$). These levels had fallen by the 6 and 24 hour time points and were no longer significantly increased when compared to control levels. In the ΣCFTE29o$^-$ cell line TNFα (10ng/ml) induced elevated levels of $PGE_2$ by the 6 hour time point ($n$ =3 $p<0.05$), as with the 9HTEo$^-$ cell line, these levels had fallen by the 24 hour time point and were no longer significantly different to their time matched controls (figure 32). Incubation of 9HTEo$^-$ cells with the cytokine mixture induced a significant increase in $PGE_2$ production at the 2, 6 and 24 hour time points ($n$ = 3 $p<0.01$). Similarly, $PGE_2$ generation by ΣCFTE29o$^-$ cells was also increased at 2, 6, and 24 hour time point in response to the mixture of cytokines ($n$ =3 $p<0.01$) (figure 33).

AA is converted into $PGE_2$ by the sequential actions of the COX and PGES enzymes. In order to investigate the effect of increasing concentrations of AA on basal $PGE_2$ generation by the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, serum starved monolayers of cells were incubated for 24 hours, at 37°C, with increasing concentrations of AA ( 0 – 10 μM). Both cell lines showed a concentration dependent increase in $PGE_2$ production in response to AA (figure 34).

NS398 is a COX-2 specific inhibitor. In order to determine the contribution of COX-2 to basal $PGE_2$ production 9HTEo$^-$ and ΣCFTE29o$^-$ cells were incubated over 2, 6, and 24 hours at 37°C with 10 μM NS398 and $PGE_2$ production by both cell lines determined (figure 35). Indomethacin inhibits both COX-1 and COX-2. Cells were incubated with 10μM indomethacin or vehicle for 6 and 24 hours at 37°C and its effect upon $PGE_2$ generation by both cell lines determined (figure 36). Surprisingly both NS398 and indomethacin failed to have an effect upon basal $PGE_2$ generation in either the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. This may be because the concentrations of these COX inhibitors used in this study were below that required for COX inhibition.

Attempts were made to investigate the production of $PGD_2$ by 9HTEo$^-$ and ΣCFTE29o$^-$ cells, but this PG was either absent or levels produced were too low to be detected by the assay used (< 5pg/ml), induction of this PGs production could not be observed at the 2, 6, 18 or 24 hour time points either basally or in response to TNFα (10ng/ml) (figure 37) or a cytomix of IL-1β (10 ng/ml), TNFα (10 ng/ml), and IFNγ (10 units/ml) (figure 38).

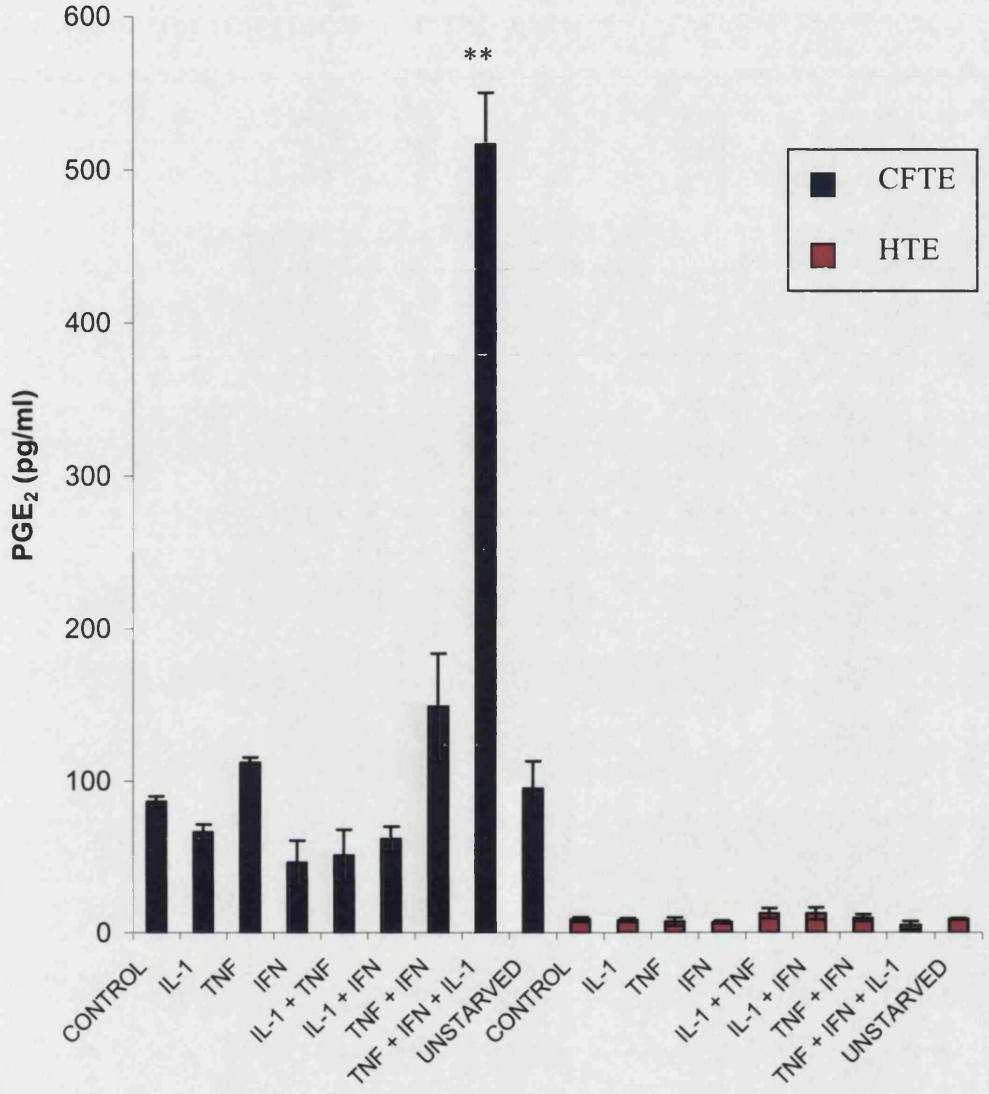



**Figure 29 $PGE_2$ production by *original batch* ΣCFTE29o⁻(1) and *original batch* 9HTEo⁻(1) cells**

Effect of 24 hour treatment with cytokines TNFα (10 ng/ml), IL-1β (10 ng/ml), and IFNγ (10 units/ml) either alone or in various combinations. The cells were serum starved for 6 hours prior to treatment. Control is the amount of $PGE_2$ produced in the presence of vehicle alone. Each bar is the mean ± SEM for 3 experiments each carried out in duplicate. (** $p<0.01$ when compared to basal)

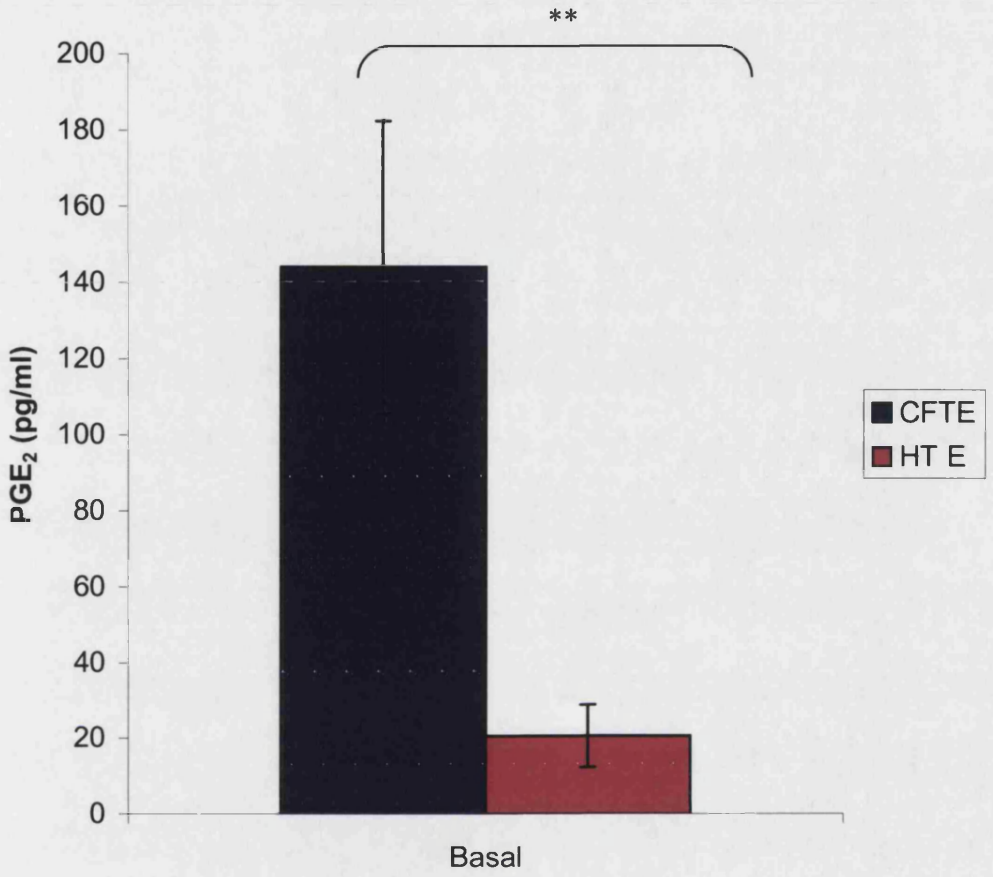



**Figure 30 Basal $PGE_2$ production by ΣCFTE29o$^-$ and 9HTEo$^-$ cells.**

The cells were serum starved for 6 hours prior to analysis. Each point in the mean ± SEM of five experiments each carried out in duplicate. (** $p<0.01$)

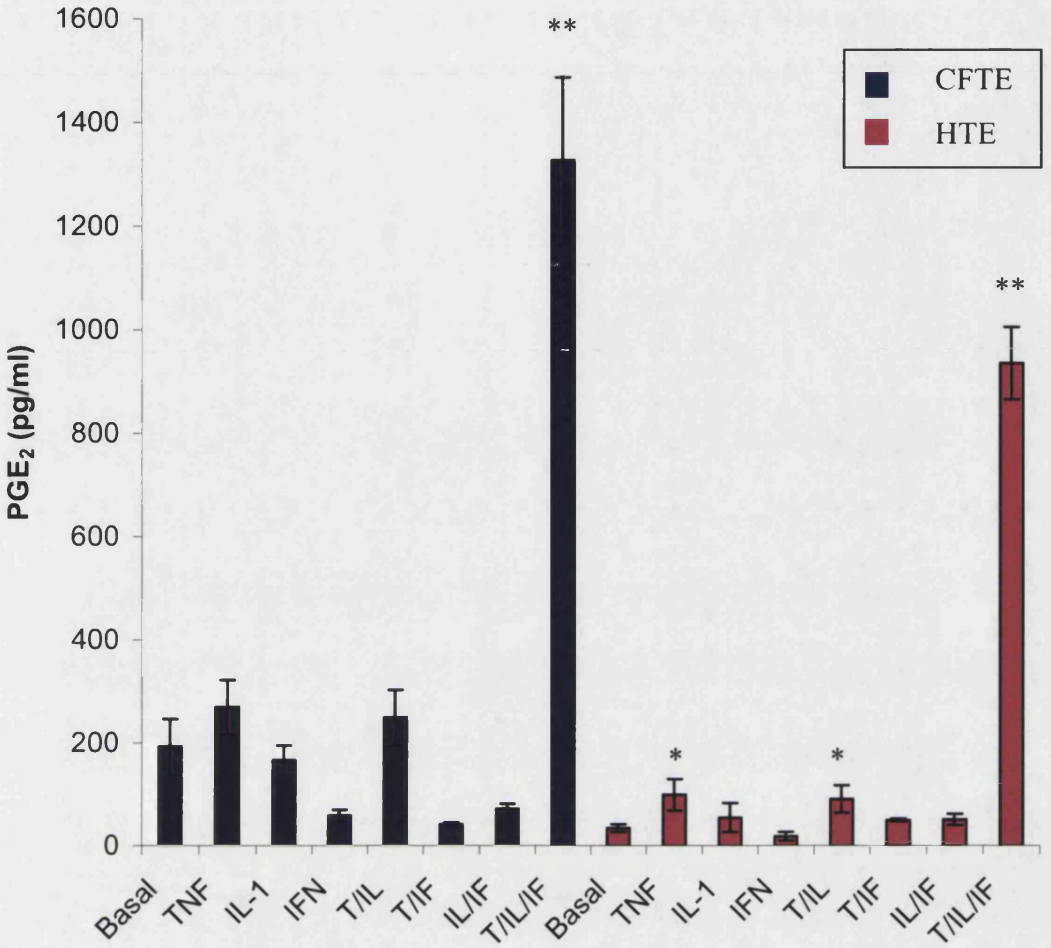



**Figure 31 Effect of cytokines on $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of 24 hour treatment with cytokines TNFα (10 ng/ml), IL-1β (10 ng/ml), and IFNγ (10 units/ml) either alone or in various combinations. The cells were serum starved for 6 hours prior to treatment. Basal is the amount of $PGE_2$ produced in the presence of vehicle alone. Each bar is the mean ± SEM for 3 experiments each carried out in duplicate. (* $p<0.05$ when compared to basal; ** $p<0.01$ when compared to basal)

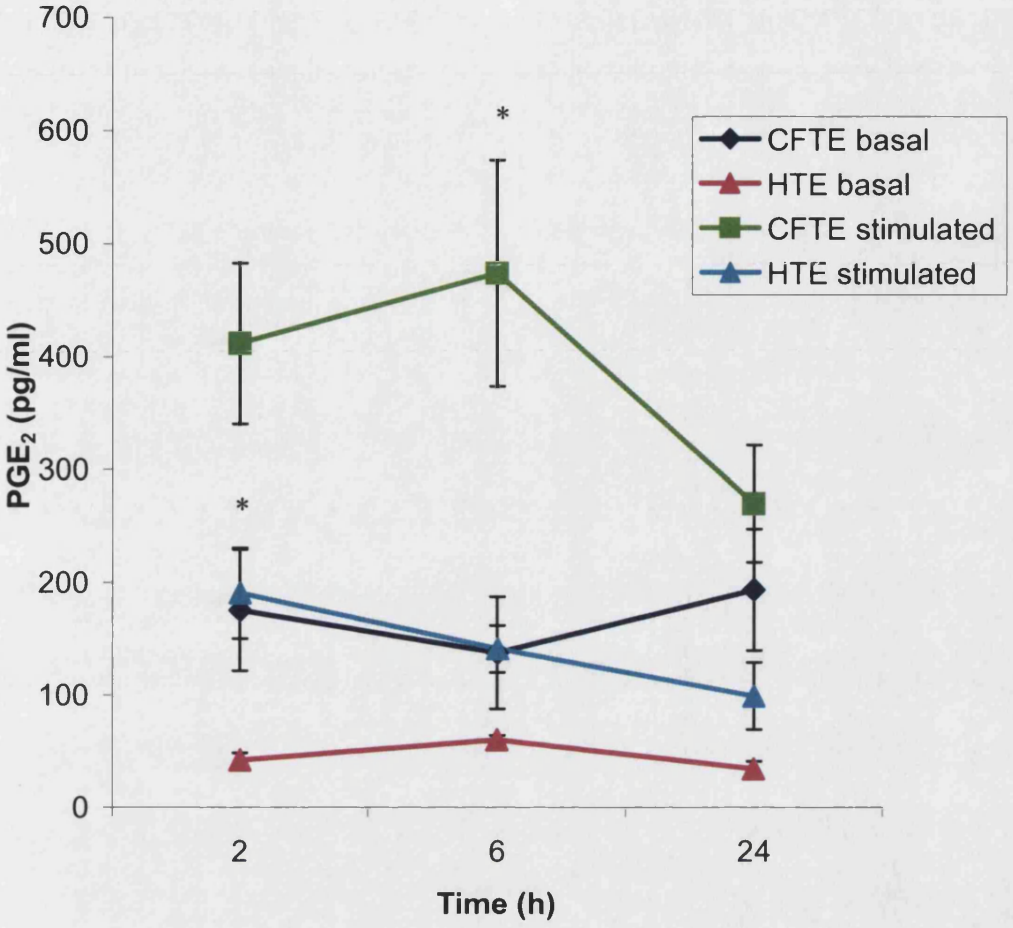



**Figure 32 Effect of TNFα on $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells**

Time course of incubation with vehicle or with TNFα (10 ng/ml). The cells were serum starved for 6 hours prior to treatment. Basal is the amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean ± SEM for 3 experiments each carried out in duplicate. (* $p<0.05$ when compared to basal)

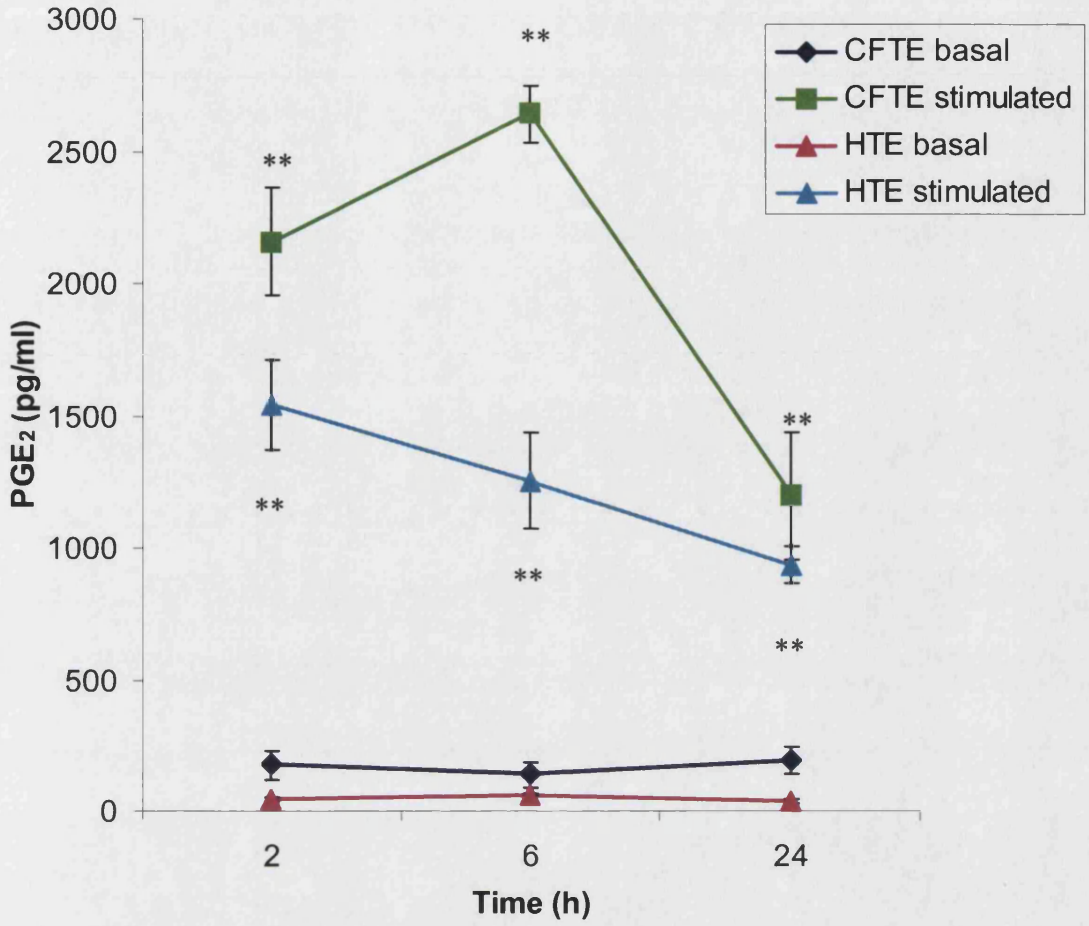



**Figure 33 Effect of cytomix on $PGE_2$ production by ΣCFTE29o$^-$ and 9HTEo$^-$ cells**

Time course of incubation with vehicle or a cytomix of TNFα (10 ng/ml), IL-1β (10 ng/ml), and IFNγ (10 units/ml). The cells were serum starved for 6 hours prior to treatment. Basal is the amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean ± SEM for 3 experiments, each carried out in duplicate. (** $p<0.01$ when compared to basal)

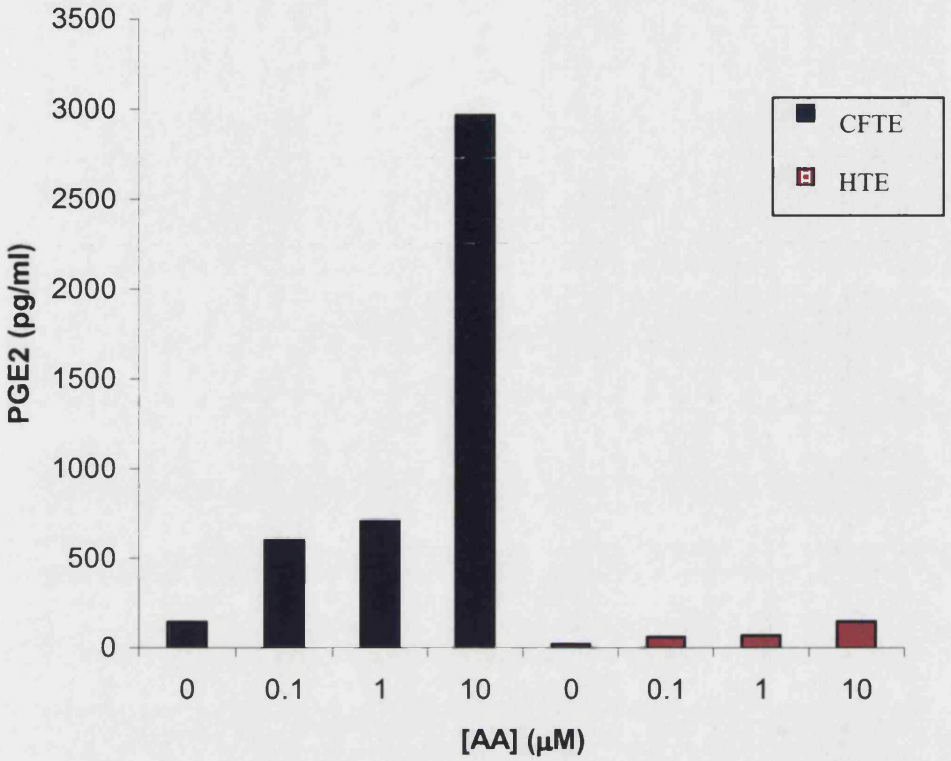



**Figure 34 Effect of AA on $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells**
Effect of 24 hour treatment with increasing concentrations of AA (0 – 10 μM). The cells were serum starved for 6 hours prior to treatment. 0 is the amount of $PGE_2$ produced in the presence of vehicle alone. Data are from duplicate measurements in a single assay.

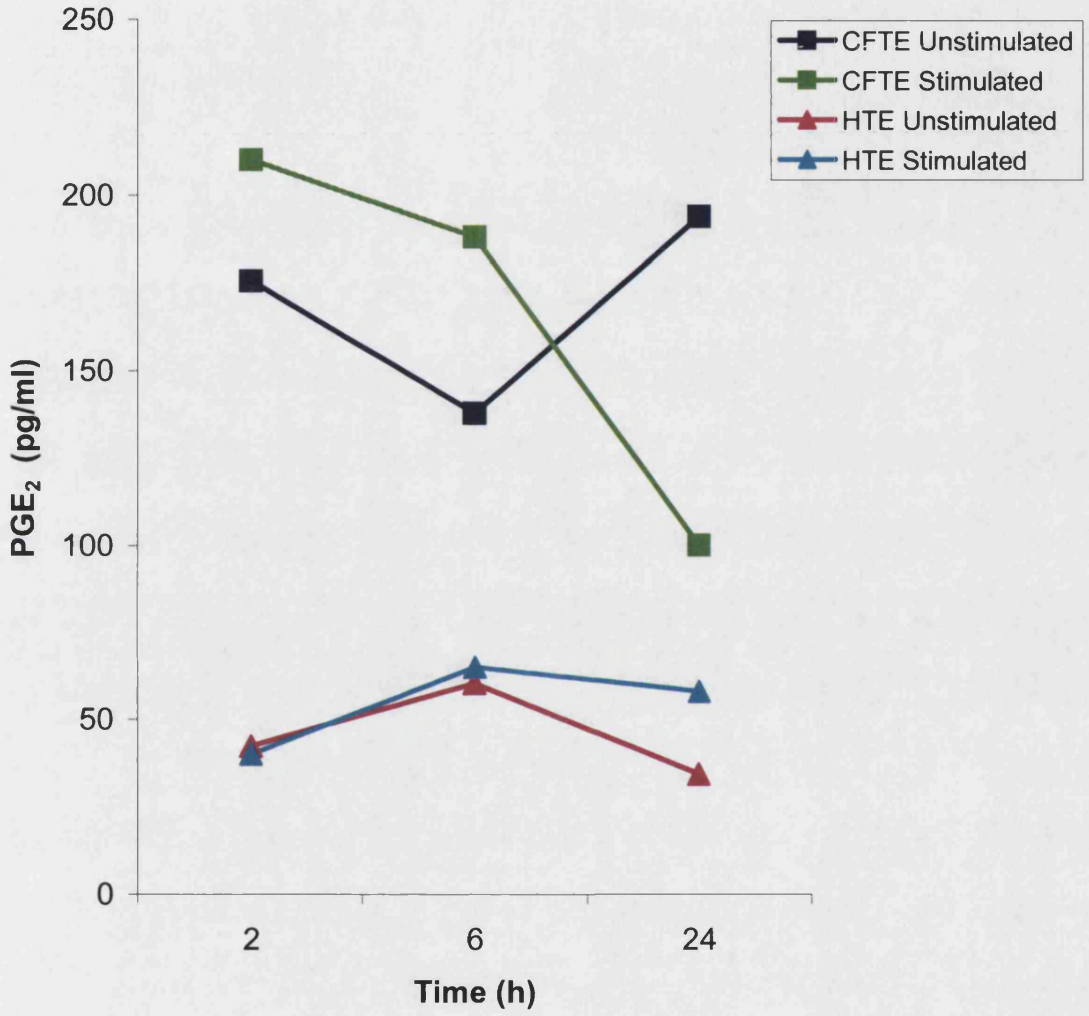



**Figure 35 Effect of NS398 on $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells**

Time course of incubation with vehicle or NS398 (10μM). The cells were serum starved for 6 hours prior to treatment. Unstimulated is the amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean of duplicate wells from one experiment.

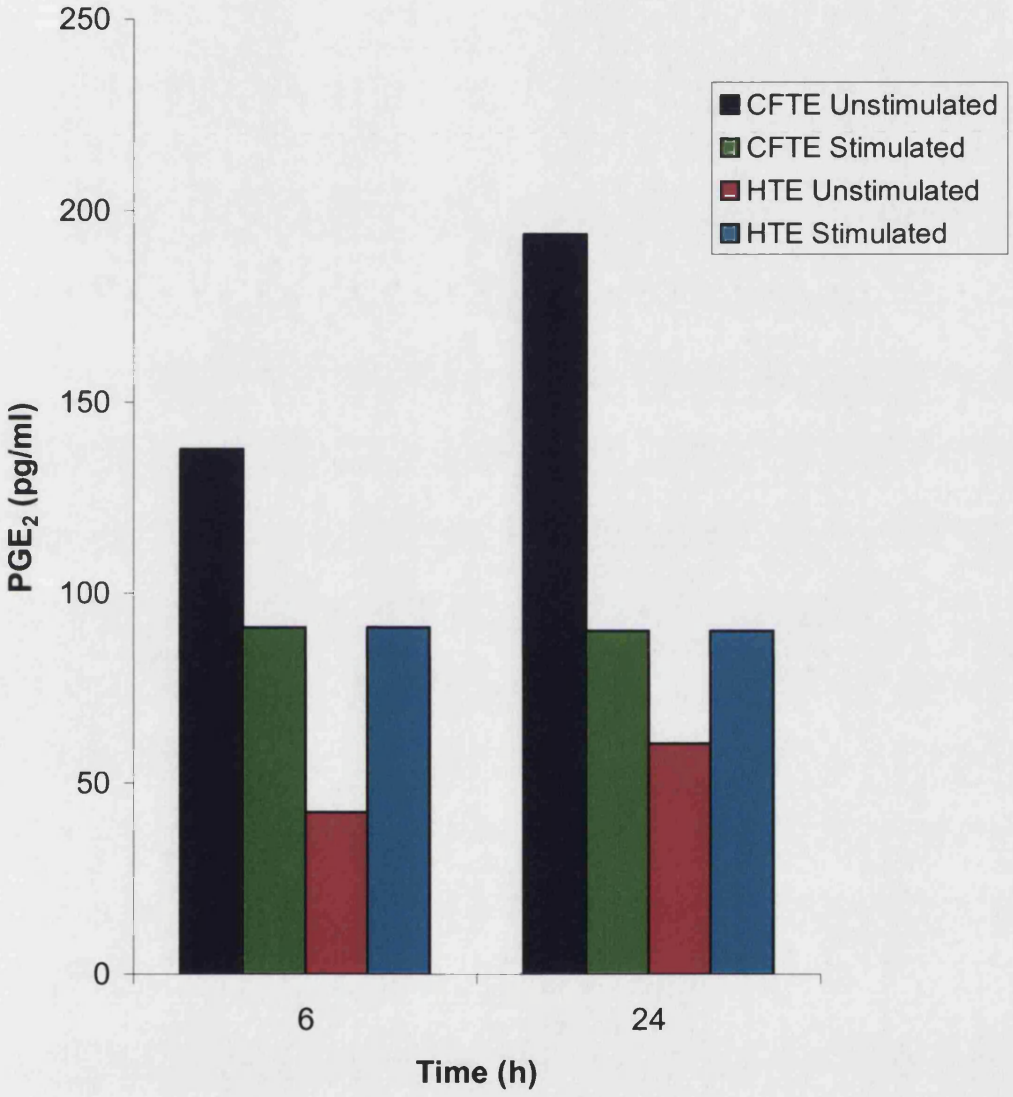



**Figure 36 Effect of indomethacin on $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of incubation with vehicle or indomethacin (10μM) at 6 and 24 h time points. The cells were serum starved for 6 hours prior to treatment. Unstimulated is the

amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean of duplicate wells from one experiment.

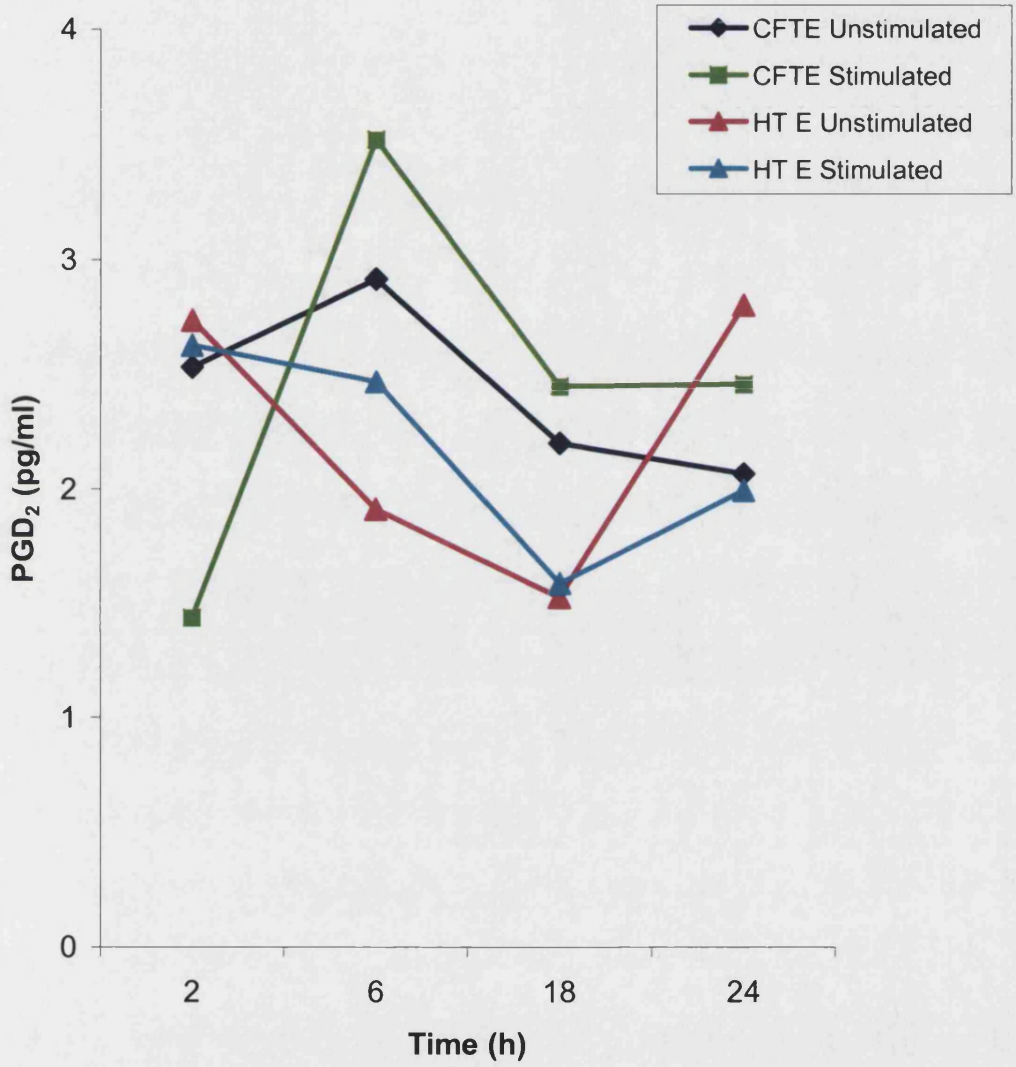



**Figure 37 Effect of TNFα on $PGD_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells**

Time course of incubation with vehicle or with TNFα (10 ng/ml). The cells were serum starved for 6 hours prior to treatment. Unstimulated is the amount of $PGD_2$ produced in the presence of vehicle alone. Each point is the mean of duplicate wells from one experiment.

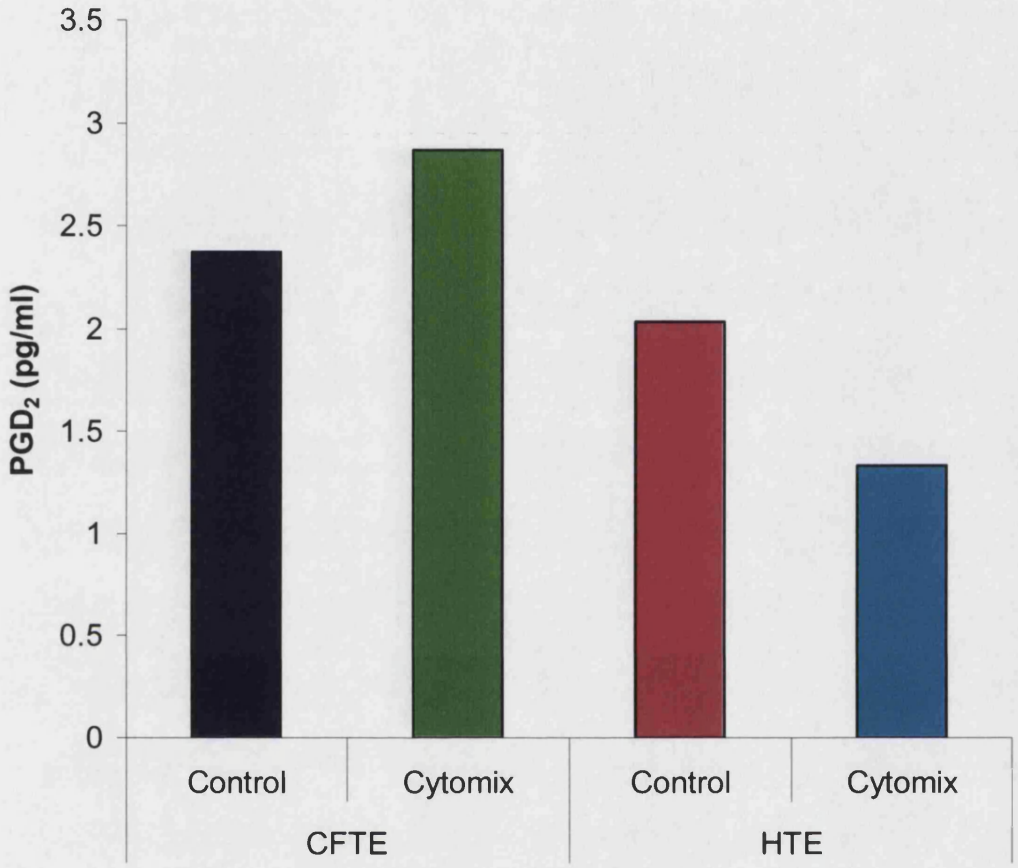



**Figure 38 Effect of cytomix on $PGD_2$ production by ΣCFTE29o$^-$ and 9HTEo$^-$ cells**

Effect of incubation with vehicle or a cytomix of TNFα (10 ng/ml), IL-1β (10 ng/ml), and IFNγ (10 units/ml) over 24 hours. The cells were serum starved for 6 hours prior to treatment. Control is the amount of $PGD_2$ produced in the presence of vehicle alone. Each point is the mean of duplicate wells from one experiment.

### 4.2.5 15–hydroxyprostaglandin dehydrogenase expression by 9HTEo$^-$ and ΣCFTE29o$^-$ cells

15–hydroxyprostaglandin dehydrogenase (PGDH) is considered the primary enzyme for prostanoid metabolism in most tissues, particularly for metabolism of $PGE_2$ (Jerde *et al.*, 2004). Ivanov and Romanovsky (2004) report that $PGE_2$ is a principal downstream mediator of fever and that in systemic inflammation levels of PGDH expression in the lungs are markedly down-regulated. This may suggest that during an inflammatory response increases in $PGE_2$ may be, in part, resultant to down regulation of its catabolic pathway. In order to investigate whether levels of PGDH may account for the differences observed in $PGE_2$ production by the cell lines used in this study, or whether the increases in $PGE_2$ production, observed in response to pro-inflammatory cytokines, may be linked to changes in PGDH protein expression, confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells, grown at 37°C, were serum starved for overnight prior to a 6 hour incubation with either vehicle or TNFα (0.1 – 10 ng/ml) and PGDH expression determined by immunoblot analysis as described in the materials and methods section. Additionally, in order to investigate whether increasing $PGE_2$ concentration is able up-regulate PGDH protein expression, confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells, grown at 37°C, were serum starved for overnight prior to a 6 hour incubation with either vehicle or $PGE_2$ (0.1 – 10 μM) and, once again, PGDH expression was determined by immunoblot analysis.

PGDH is constitutively expressed at similar levels in both 9HTEo$^-$ and ΣCFTE29o$^-$ cells (figure 39). No change in PGDH expression was detectable in either cell line, when compared to basal, in response to stimulation over 6 hours with increasing concentrations of pro-inflammatory cytokine TNFα (0.1 – 10ng/ml) (figure 40). Similarly, no change in PGDH expression was detectable, in comparison to basal, in either 9HTEo$^-$ or ΣCFTE29o$^-$ cells in response to incubation with increasing concentrations of $PGE_2$ (0.1 – 10nM) (figure 41).

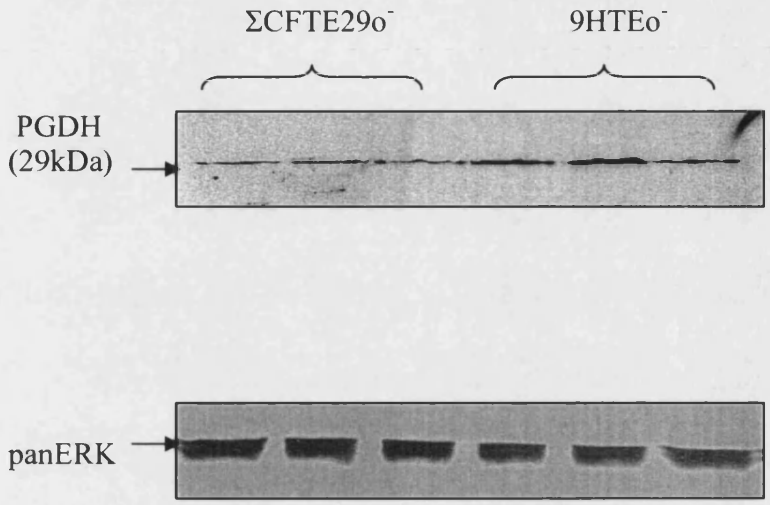



**Figure 39 Basal expression of PGDH protein in ΣCFTE29o⁻ and 9HTEo⁻ cells**

Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to harvest. The top panel is the immunoblot probed with a specific antibody against PGDH, lanes 1 – 3 are unstimulated ΣCFTE29o⁻ samples from 3 separate experiments; Lanes 4 – 6 are unstimulated 9HTEo⁻ samples from 3 separate experiments.. This immunoblot is representative of three others. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

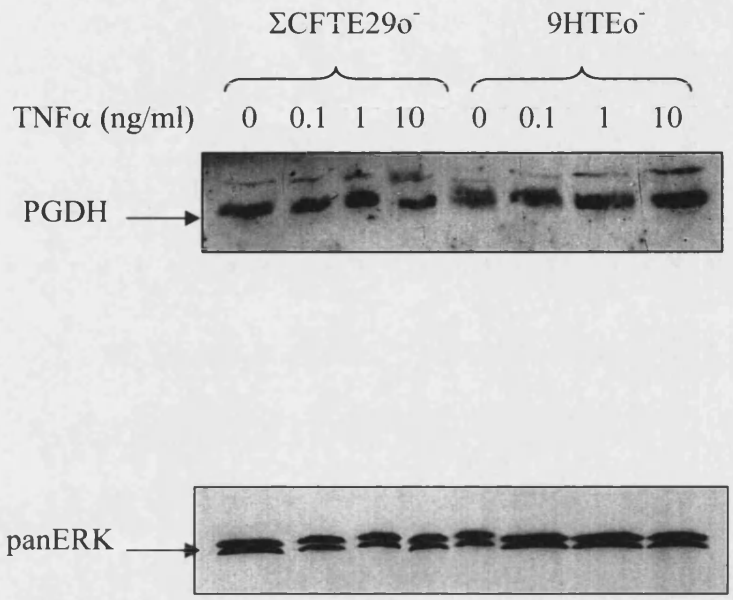



**Figure 40 Effect of TNFα on expression of PGDH protein in ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of exogenous application of increasing concentrations of TNFα (0.1 - 10 ng/ml), over 6 hours. Western blot analysis of whole cell lysates. Cells were serum starved overnight prior to treatment. The top panel is the immunoblot probed with a specific antibody against PGDH, this immunoblot is representative of two others. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

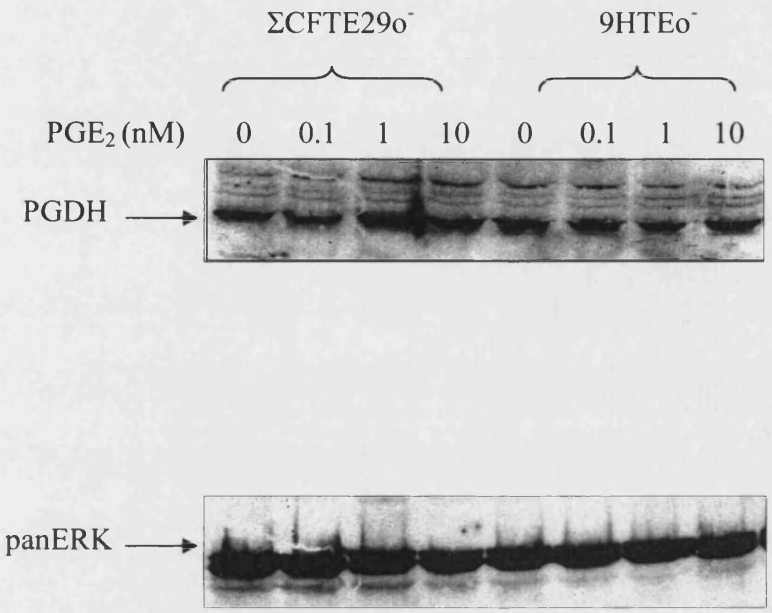



**Figure 41 Effect of $PGE_2$ on expression of PGDH protein in ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of exogenous application of increasing concentrations of $PGE_2$ (0.1 - 10 nM), over 6 hours. Western blot analysis of whole cell lysates. Cells were serum starved overnight prior to treatment. The top panel is the immunoblot probed with a specific antibody against PGDH, this immunoblot is representative of two others. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

### 4.2.6 Metabolic activity of human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$

In order to determine that differences observed in protein expression and $PGE_2$ production between the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines are not a result of differences in the basic metabolic rate of the cell lines, monolayers of cells, approximately 90% confluent, were serum starved for 24 hours and MTT assays performed as detailed in the materials and methods section. Additionally, MTT assays were conducted on cells stimulated with cytomix and increasing concentrations of TNFα, at 6 and 24 hour time points, in order to ascertain that the decrease in $PGE_2$ production observed at the 24 hour time point in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines was not a result of a reduction in the cellular viability as a result of serum starvation and / or cytokine stimulation.

No difference in metabolic rate could be detected between 9HTEo$^-$ cells (87.8 ± 10.7 % max absorbance (550nm)) and ΣCFTE29o$^-$ cells (100 ± 8.1 % max absorbance (550nm)) after 6 hours of serum starvation (figure 42). The cells were serum starved for 6 hours prior to stimulation with increasing concentrations of TNFα (0.01 - 10 ng/ml) for 6 hours (figure 43) and 24 hours (figure 44). No significant change in the cells activity could be detected in either cell line at either time point or in response to any of the different concentrations of TNFα used in this study. Additionally, no change in metabolic activity was apparent in response to the cytokine mixture of TNFα (10ng/ml), IL-1β, (10 ng/ml) and IFNγ (10 units/ml) in either the 9HTEo$^-$ or the ΣCFTE29o$^-$ cell line (figure 45).

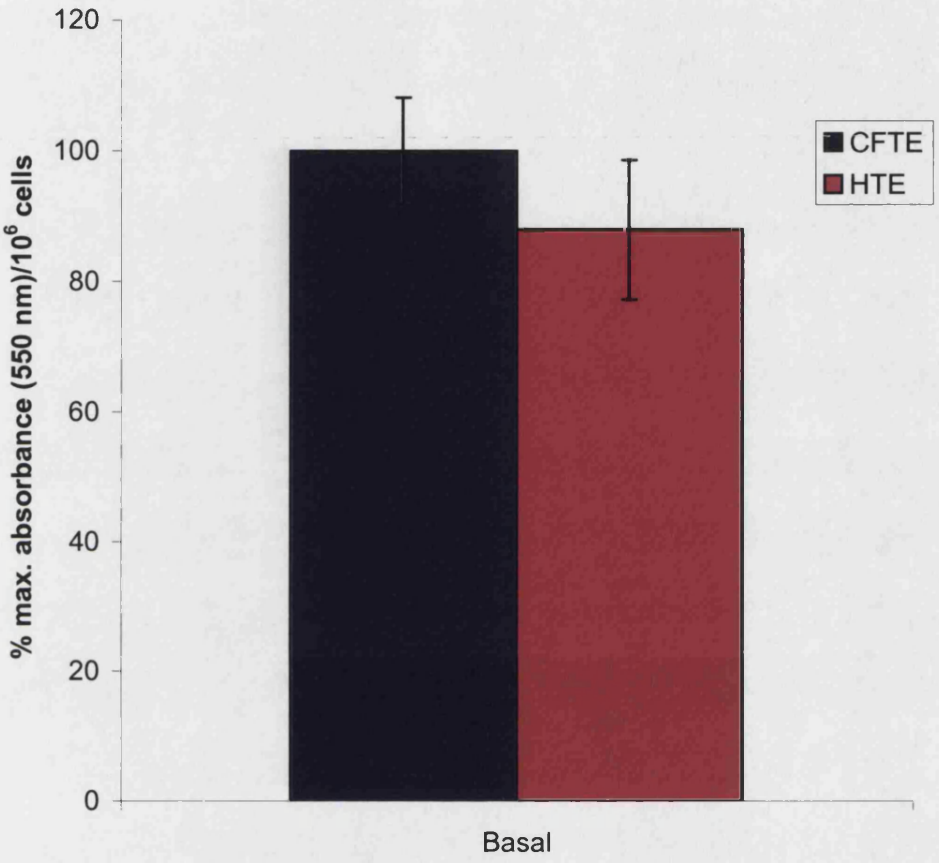



**Figure 42 Relative basal metabolic activity of 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 hours in serum free medium, as measured by MTT assay.**

The cells were serum starved for 6 hours prior to analysis. Basal is the absorbance measured at a wavelength of 550 nm for unstimulated cells. Each bar is the mean ± SEM expressed as a percentage of the maximum absorbance for 5 experiments carried out in triplicate.

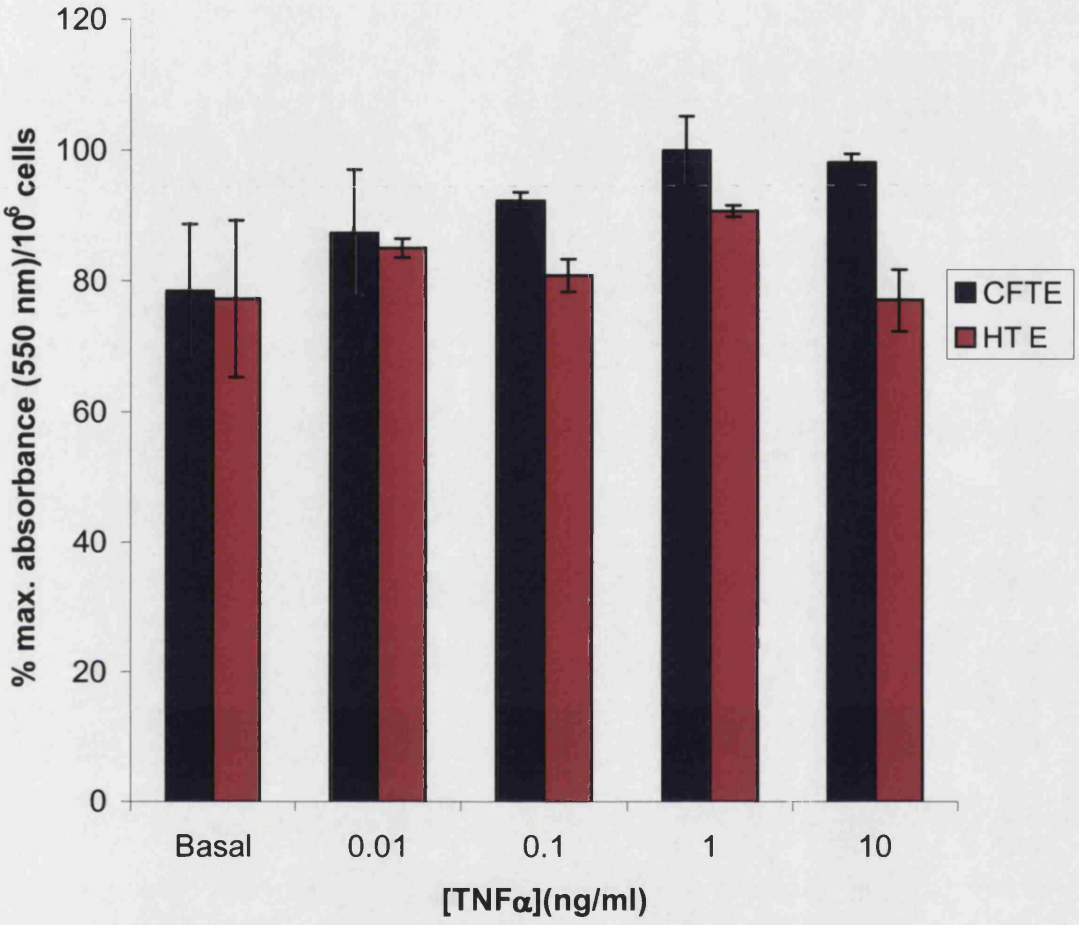



**Figure 43 Relative basal metabolic activity of 9HTEo⁻ and ΣCFTE29o⁻ - Effect of TNFα**

Effect of increasing concentrations of TNFα (0.01 – 10 ng/ml) over 6 hours, as measured by MTT assay. The cells were serum starved for 6 hours prior to analysis. Basal is the absorbance measured at a wavelength of 550 nm for cells stimulated with vehicle alone. Each bar is the mean ± SEM expressed as a percentage of the maximum absorbance for 3 experiments each performed in triplicate.

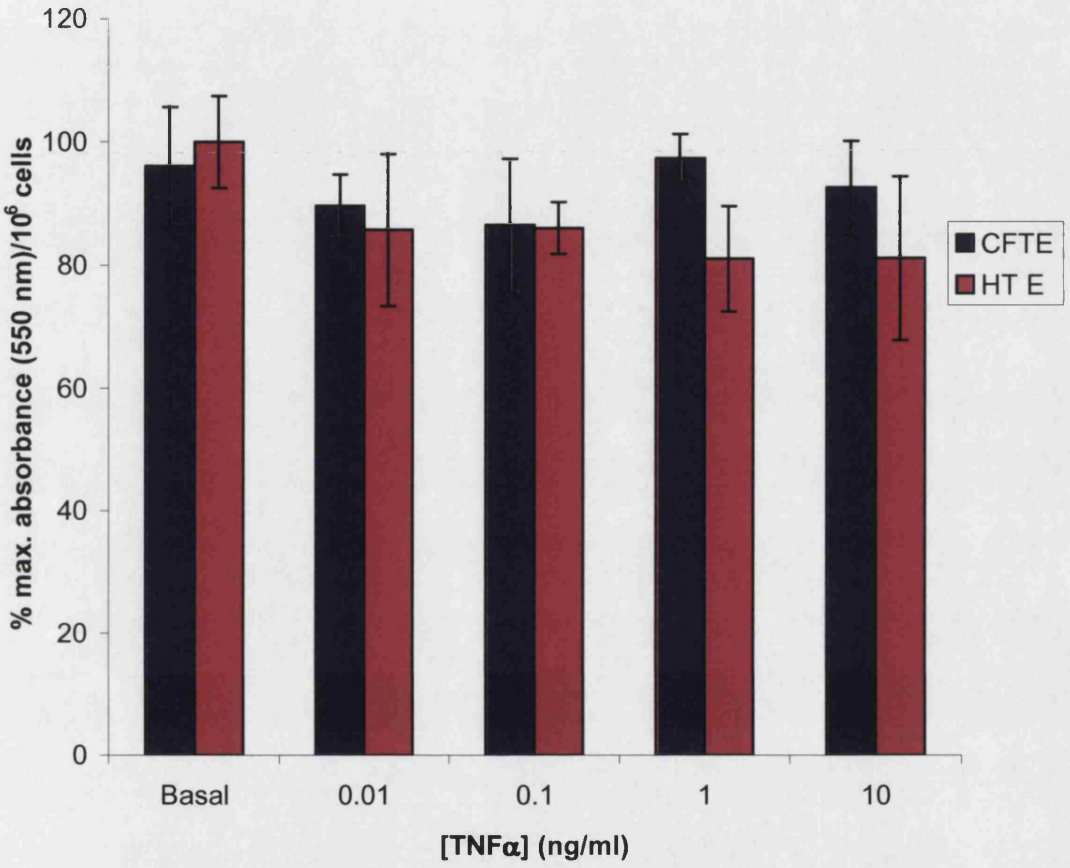



**Figure 44 Relative basal metabolic activity of 9HTEo⁻ and ΣCFTE29o⁻ in response to increasing concentrations of TNFα (0 – 10 ng/ml) over 24 hours, as measured by MTT assay**

The cells were serum starved for 6 hours prior to analysis. Basal is the absorbance measured at a wavelength of 550 nm for cells stimulated with vehicle alone. Each bar is the mean ± SEM expressed as a percentage of the maximum absorbance for 3 experiments each carried out in triplicate.


**Figure 45 Relative basal metabolic activity of 9HTEo⁻ and ΣCFTE29o⁻ - Effect of cytomix**

Effect of increasing concentrations of a cytomix of TNFα (10 ng/ml), IL-1β (10 ng/ml), and IFNγ (10 units/ml) over a period of 24 hours on basal metabolic actvity. As measured by MTT assay. The cells were serum starved for 6 hours prior to analysis. Basal is the absorbance measured at a wavelength of 550 nm for cells stimulated with vehicle alone. Each bar is the mean ± SEM for 3 experiments each carried out in triplicate

## 4.3 Discussion

Prostaglandins (PGs) are generated under both physiological and pathophysiological conditions and regulate many aspects of airway physiology. Airway epithelial cells respond to inflammatory insults by increasing production of cytokines such as TNFα, IL-1β, and IFNγ which, in turn, regulate the production of pro-inflammatory mediators, such as PGs, by inducing the expression of their synthesising enzymes (Mitchell *et al*, 1994). One of the hallmarks of CF airway disease is a persistent and exaggerated inflammatory response that leads to destruction of the host tissue and ultimately pulmonary failure. Escotte *et al.,* 2002 report that this exaggerated inflammatory response is associated with an abnormal increase in the production of pro-inflammatory cytokines. Furthermore, a number of studies report that PG levels in the sputum of CF patients are elevated when compared to healthy controls and may participate in the pathophysiology of the disease (Zakrzewski *et al.,* 1987; Strandvik *et al.,* 1996). It is postulated that the induction and regulation of COX-2 may be a crucial element in inflammatory disorders of the airways such as cystic fibrosis where a chronic inflammatory response leads to destruction of host tissue. $PGE_2$ stimulates mucus secretion and oedema, characteristics commonly found in the CF lung, and is reported to synergise with IL-8 to enhance neutrophil migration (Smith *et al,* 2002). The constitutive expression of COX-2 by airway epithelial cells and the ability of PGs to act as both positive and negative regulators of inflammation may suggest a homeostatic role for COX-2 products in the airways. Therefore, elucidation of the role and regulation of PG production by airway epithelial cells may provide insight into more effective therapeutic approaches to regulate inflammatory disorders of the airways such as CF. The work in this study investigates the regulation of $PGE_2$ production in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$, cells of a non-CF and CF phenotype respectively. Attempts were also made to investigate PG production in genetically matched CF and non-CF phenotype cell lines, however the data obtained from these cell lines were unreproducible and were not included in this study.

Both COX-1 and COX-2 proteins are reported to be constitutively expressed in airway epithelial cells (Mitchell *et al.,* 1994; Asano *et al,* 1996; Watkins *et al.,* 1999; Rodgers *et al.,* 2002). Consistent with these findings this study reports that both COX-1 and COX-2 proteins are expressed in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$ in the absence of cytokine stimulation. While no difference between COX-2 protein expression could be detected between these cell lines, 9HTEo$^-$ cells expressed more COX-1 protein than ΣCFTE29o$^-$ cells. A number of studies report that IFNγ, TNFα, and IL-1β induce COX-2 protein expression in airway cells (Mitchell *et al.,* 1994; Belvisi *et al.,* 1997; Pang *et al.,* 1998; Bonazzi *et al.,* 2000) while IL-13 inhibits COX-2 expression (Berg *et al.,* 2000). In the study reported here, stimulation of the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines over 24 hours with pro-inflammatory cytokines IFNγ, TNFα, and IL-1β either alone or in combination had no effect upon COX-1 or COX-2 protein expression. Additionally, IL-13 had no effect on COX-2 protein expression in either cell line. At the 48 hour time point microscopic examination of the cells revealed a decrease in cell number and an increase in cell debris, particularly affecting the 9HTEo$^-$ cell line. At all other time points cell viability was much higher (approximately 85%). In light of this, stimulation of these cells was routinely carried out over a 24 hour time course. As COX-1 is a housekeeping enzyme the inability of pro-inflammatory cytokines to induce its expression was to be expected. However, the inability of these cytokines, either alone or in combination, to induce COX-2 protein expression was of more interest.

In order to be certain that the lack of COX-2 induction by cytokines was not a result of the cytokines being non-viable, IL-8 ELISAs were conducted on supernatants from cells stimulated with increasing concentrations IFNγ, TNFα, and IL-1β either alone or in combination. IL-8 is a potent neutrophil chemoattractant that has been implicated in a number of inflammatory airway diseases such as cystic fibrosis. IL-8 has been reported to be up-regulated in response to pro-inflammatory cytokines in a number of airway epithelial cell lines (Schweibert *et al.*, 1999). In this study, both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines produced a small amount of IL-8 in the absence of cytokine stimulation; this finding is consistent with other reports (Aldallal *et al.,* 2002; Becker *et a.,* 2004). IL-8 generation was seen to increase in response to TNFα and IL-1β either

alone or in combination. Maximal induction of IL-8 was observed in response to a combination of TNFα, IL-1β, and IFNγ. However, IFNγ alone had no effect on basal IL-8 production. The lack of IL-8 induction by IFNγ alone, and the apparent synergy between TNFα, IL-1β and IFNγ has previously been observed in the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines (Lavelle, 2002 unpublished data). However, from the data in this study it is not possible to speculate on the mechanisms behind the cooperative effect of these cytokines. The induction of IL-8 production by both cell lines used in this study confirmed the viability of the cytokines and their signal transduction pathways.

$PGE_2$ is the most common PG in the airways and is believed to be predominantly a COX-2 product. The major finding of this study was that the CF-phenotype cell line ΣCFTE29o$^-$ produced significantly higher levels of $PGE_2$ under basal conditions than the non-CF phenotype cell line 9HTEo$^-$ ($p<0.01$). Despite these cell lines not being a genetically matched pair, the difference in $PGE_2$ levels observed between the CF and non-CF phenotype cell lines is supported by the findings of Zakrzewski *et al.,* 1987 and Strandvik *et al.,* 1996 who report that PG levels are elevated in the sputum of CF patients when compared to healthy controls. Additionally, Freedman *et al,* 1999 and 2002 report the presence of a membrane lipid defect in the lung, pancreas and ileum from *cftr*$^{-/-}$mice that is characterised by an increase in phospholipid-bound AA and an increase in levels of $PGE_2$ in the BAL fluid from *cftr*$^{-/-}$ mice when compared to wild-type controls. Attempts were made to investigate the production of $PGD_2$ by 9HTEo$^-$ and ΣCFTE29o$^-$ cells, but this PG was either absent or levels produced were too low to be detected by the assay used. $PGD_2$ levels are reported to be elevated in the rat carrgeenin-induced pleurisy model of inflammation at a 48 hour time point (Gilroy *et al.,* 1999). However, as cells were not determined to be viable at the 48 hour time point $PGD_2$ levels at this time point could not be investigated in this study.

Interestingly, $PGE_2$ has been demonstrated to promote the inherent activity of the p65/RelA subunit of NF-κB, strongly synergising with TNFα promoting NF-κB dependent gene transcription (Poligone and Baldwin, 2001). However, despite basal levels of $PGE_2$ produced by the ΣCFTE29o$^-$ cell line being approximately seven times

those of the 9HTEo$^-$ cell line no difference in the level of IL-8 produced under basal conditions by these cell lines could be detected. ΣCFTE29o$^-$ cells produced more IL-8 in response to high concentrations of TNFα ($p<0.05$) than 9HTEo$^-$ cells, however, the CF-phenotype cells produced significantly less IL-8 than the non-CF cell line in response to high levels of IL-1β. It is possible that these differences are a result of differences between the cell lines' pattern of receptor expression, but from the data gathered in this study it is difficult to speculate on the mechanisms behind these differences. Additionally, $PGE_2$ and AA are reported to induce IL-8 in airway epithelial cells (Rodgers *et al.,* 2002). However, in this study no change in IL-8 production could be observed in response to either $PGE_2$ or AA.

Despite the inability of cytokines to induce COX-2 protein expression, stimulation of the cell lines with TNFα resulted in an increase in $PGE_2$ generation at the 2 hour time point in 9HTEo$^-$ cells ($p<0.05$) and at the 6 hour time point in ΣCFTE29o$^-$ cells ($p<0.05$). Levels in both cell lines were not determined to be different from basal by the 24 hour time point. A similar pattern of induction was observed in response to a combination of IFNγ, TNFα, and IL-1β, however the increase observed in $PGE_2$ generation was significant at 2, 6, and 24 hours in both cell lines ($p<0.01$) and was significantly higher than levels generated in response to TNFα alone ($p<0.01$). The rapid increase in $PGE_2$ generation in response to pro-inflammatory stimuli is reported to occur in the rat carrgeenin-induced pleurisy model of inflammation and is associated with maximal COX-2 protein expression at the 2 hour time point, COX-1 protein expression remaining constant. Furthermore, this study reports that COX-2 protein expression increases again at the 48 hour time point and is associated with and increase in $PGD_2$ production thought to be involved in the resolution of the inflammatory response (Gilroy *et al.,* 1999). The role of PGs in the airways is complex. $PGE_2$ is reported to have both pro-inflammatory and anti-inflammatory effects dependent upon the receptor sub-type it activates, the time point of its production and the tissue that it is acting upon. The induction of $PGE_2$ production by the cell lines used in this study, in response to pro-inflammatory cytokines, at the 2 and 6 hour time points suggests that $PGE_2$ plays a role in the initial phase of the inflammatory response. High basal expression of $PGE_2$ by the ΣCFTE29o$^-$ cell line compared to the 9HTEo$^-$ cell line is

also consistent with the pro-inflammatory phenotype of CF cells and highlights the airway epithelium as a contributor to the inflammatory response and as a therapeutic target in airway diseases, such as CF, where inflammation plays a key role. The inability of cytokines to induce COX-2 at any time point observed in this study suggests that the increase in levels of $PGE_2$ generated in response to pro-inflammatory cytokines may be resultant of either an increase in the activity of the COX enzymes or a change in expression and / or activity of PGE synthase enzymes.

The conversion of $PGH_2$ to $PGE_2$ is catalysed by PGE synthase. Three major isoforms of PGES have been identified to date. cPGES a constitutively expressed isoform reported to act in concert with COX-1 (Murakami *et al,* 2000), mPGES acts in concert with COX-2 and is inducible by cytokines (Murakami *et al,* 2000), and mPGES-2 a constitutively expressed isoform that promotes $PGE_2$ production via both COX-1 and COX-2 (Murakami *et al.,* 2003). At the time this study was conducted antibodies were only available for cPGES and mPGES and so expression of mPGES-2 was not investigated. cPGES was constitutively expressed by both 9HTEo$^-$ and ΣCFTE29o$^-$ cells, as expected for a housekeeping enzyme its expression was not up-regulated by pro-inflammatory cytokines. Interestingly, mPGES protein was not detectable in either tracheal epithelial cell line. A549 cells were used as a positive control to demonstrate that mPGES protein was detectable under the conditions used in this study and that mPGES protein expression can be elevated in response to TNFα. A lack of mPGES expression paired with high basal expression of both cPGES and COX-2 has also been observed in cultured KAT-50 thyrocytes (Han and Smith, 2002). The authors of this report postulate that low $PGE_2$ production by this cell line, despite high levels of COX-2 protein being expressed, may be a result of inefficient coupling between cPGES and COX-2 in the absence of mPGES. In this study $PGE_2$ only appears to be low in the 9HTEo$^-$ cell line, despite this cell line expressing more COX-1 protein than the ΣCFTE29o$^-$ cell line. This may be a result of more efficient coupling of cPGES and mPGES by the ΣCFTE29o$^-$ cells or it may be a consequence of differences in expression of mPGES-2 between these cells. However, from the data gathered in this study it is not possible to reach a definite conclusion.

As the PGES enzymes are glutathione (GSH) dependent requiring glutathione for optimal activity (Murakami *et al.,* 2000), basal GSH levels were investigated in ΣCFTE29o$^-$ and 9HTEo$^-$ cells in order to determine whether differences in levels of glutathione could be affecting $PGE_2$ production. Additionally, GSH production in CF airways is reported to be deficient compared to production by non-CF airways (Roum *et al.,* 1999). It is therefore of interest to investigate whether these differences are observable in CF and non-CF cell lines. Under the conditions used in this study, no difference could be detected in basal GSH levels between the CF and non-CF cell lines. Consequently differing GSH levels could be ruled out as a factor contributing to the differences in $PGE_2$ production observed.

As no difference in the expression of $PGE_2$ synthesising enzymes could be detected to explain the difference in $PGE_2$ production by the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, the expression of $PGE_2$ metabolising enzyme PGDH was investigated. PGDH is considered the primary source of PG metabolism in most tissues, particularly metabolism of $PGE_2$ but mechanisms regulating its expression are poorly understood (Jerde *et al.,* 2004). If the 9HTEo$^-$ cell line constitutively expressed higher levels of this protein than the ΣCFTE29o$^-$ cell line, it may have explained why $PGE_2$ levels were so different. However, PGDH was found to be constitutively expressed in both airway epithelial cell lines at similar levels, therefore differences in the expression of this enzyme can not be held accountable for the differences in $PGE_2$ observed in this study. Furthermore, PGDH protein expression could not be induced by stimulation of these cell lines with cytokines, this may be a result of maximal expression of PGDH being present under basal conditions as a consequence of constitutive production of $PGE_2$ by the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Whether PGDH has a higher level of activity in the 9HTEo$^-$ cell line was not investigated in this study.

As no differences in the expression of enzymes involved in the production or metabolism of $PGE_2$ were detected, MTT assays were conducted to determine the basal metabolic rates of these cells. If the ΣCFTE29o$^-$ cells proved to be basally more active

than the 9HTEo$^-$ cells this may to some extent have provided explanation for the higher levels of $PGE_2$ produced by the CF cell line. However, basal metabolic activity was not significantly different between the two cell lines. Incubation of the cells with pro-inflammatory cytokines did not affect the basal metabolic activity of either cell line, even at concentrations and time points that induced increases in $PGE_2$ and IL-8 generation. It is possible that these cells are constantly in a high state of metabolic activity producing factors such as IL-8 and $PGE_2$ under basal conditions as a primary defence mechanism against external insults. The tracheal epithelium is constantly in contact with inhaled air and pathogens, it may be that expression of enzymes involved in the generation of an inflammatory response are constitutively expressed at maximal concentrations as a consequence of continuous exposure to low-level environmental stimuli.

As previously stated, $PGE_2$ is a product of the actions of COX and cPGES enzymes. Furthermore, it is reported that $PGE_2$ participates in the regulation of COX-2 protein expression and subsequently modulates further PG production (Faour *et al,* 2001; Poligone and Baldwin, 2001). However, little is known about the feedback effects of $PGE_2$ on COX, PGES and PGDH enzymes in the tracheal epithelium. In this study, exogenously applied $PGE_2$ had no effect upon COX-2, cPGES, mPGES or PGDH protein expression in either 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines. This may indicate that $PGE_2$ does not exert an effect upon its metabolic pathway in this cell line. Alternatively the constitutive expression of PGDH by these cell lines may result in inactivation of $PGE_2$ before it can exert an effect upon this pathway. Furthermore, Pang *et al.,* 2003 reports that COX inhibitors indomethacin and NS-398 are able to modulate COX-2 protein expression in a cell specific manner. In this study, these NSAIDs had no effect upon COX-2 protein expression in the presence or absence of TNFα.

In conclusion, the data presented here demonstrate that COX-2 is constitutively expressed in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$. Consistent with reports that PG production by CF airways is elevated when compared to that of non-CF airways (Zakrzewski *et al.,* 1987; Strandvik *et al.,* 1996), this study reports that

CF-phenotype ΣCFTE29o$^-$ cells produce significantly more $PGE_2$ than non-CF phenotype 9HTEo$^-$ cells. No elevation in enzymes from the biosynthetic pathway could be observed in ΣCFTE29o$^-$ cells compared to 9HTEo$^-$ cells nor could any difference in PGDH expression be observed suggesting that differences in $PGE_2$ production by the cell lines may be a result of differences in the activity of the enzymes present or differences in substrate levels. The latter hypothesis would be consistent with the findings of Freedman *et al.,* 1999, who report the presence of a membrane lipid imbalance in the lungs of *cftr*$^{-/-}$ mice characterised by elevated levels of phospholipid-bound AA. The high basal expression of the enzymes of the $PGE_2$ synthetic pathway by tracheal epithelial cell lines, and the rapid induction of $PGE_2$ production by pro-inflammatory cytokines suggests that $PGE_2$ expression by the tracheal epithelium may be part of a natural defence mechanism as well as a contributor to airway inflammation.

## 4.3 Summary of Results

- The 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines produce low basal levels of IL-8 which can be elevated by incubation with pro-inflammatory cytokines demonstrating that these cell lines express functional receptors for cytokines
- Both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell line constitutively express COX-1 protein with 9HTEo$^-$ cells producing more COX-1 than ΣCFTE29o$^-$ cells
- Stimulation of the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines with pro-inflammatory cytokines did not result in elevation of COX-1 protein expression
- COX-2 protein is constitutively expressed by both 9HTEo$^-$ and ΣCFTE29o$^-$ cells, there is no detectable difference between the levels of COX-2 expressed in these cells lines
- Basal COX-2 protein expression is not affected by incubation with pro-inflammatory cytokines in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines at any time point from 2 – 48 hours
- NSAIDs do not effect COX-2 protein expression in 9HTEo$^-$ or ΣCFTE29o$^-$ cells and at the concentrations used in this study failed to inhibit $PGE_2$ production by either cell line
- Exogenously applied $PGE_2$ did not have any effect on COX-2 protein expression
- cPGES is constitutively expressed at a similar level in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines and is not up-regulated by pro-inflammatory cytokines or exogenously applied $PGE_2$
- mPGES protein is constitutively expressed in the A549 cell line and its expression is elevated by TNFα
- mPGES protein is not detectable in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines under basal conditions or upon stimulation with TNFα

- The 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines produce $PGE_2$ under basal conditions with the ΣCFTE29o$^-$ cells producing approximately seven times more $PGE_2$ than the 9HTEo$^-$ cells
- $PGE_2$ generation can be induced by incubation of the cells with pro-inflammatory cytokines. Maximal induction of $PGE_2$ in response to cytokines occurs at the 2 hour time point in 9HTEo$^-$ cells and 6 hour time point in ΣCFTE29o$^-$ cells
- Exogenous application of AA induces a concentration dependent increase in $PGE_2$ generation by both cell lines
- PGDH is expressed in 9HTEo$^-$ and ΣCFTE29o$^-$ cells in the absence of cytokine stimulation.
- Incubation of the cell lines with cytokines or $PGE_2$ did not effect basal PGDH expression
- No difference in the basal metabolic rate of 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines could be detected
- Cytokines do not appear to have a significant effect upon the metabolic activity of either cell line

# Chapter 5: The role of Peroxisome proliferator-activated receptors and ligands in the regulation of prostaglandin production in human tracheal epithelial cell lines

## 5.1 Introduction

Airway epithelial cells are reported to express constitutively high levels of PPARγ and it is postulated that one of its roles in the airways may be that of a negative regulator of inflammation (Wang *et al.*, 2001). The aim of this work was to study the expression of PPARγ in non-CF and CF phenotype cell lines 9HTEo$^-$ and ΣCFTE29o$^-$ and to investigate the role of PPARγ ligands in the regulation of the $PGE_2$ metabolic pathway.

## 5.2 Results

### 5.2.1 Expression of PPARγ in 9HTEo$^-$ and ΣCFTE29o$^-$ cells

In order to determine basal expression of PPARγ in the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, confluent monolayers of cells were serum starved for 6 hours at 37°C and PPARγ protein was determined using immunoblot analysis, as described in the materials and methods section. ΣCFTE29o$^-$ cells express high levels of PPARγ. 9HTEo$^-$ cells also express PPARγ but at a lower level than observed in the ΣCFTE29o$^-$ cell line (9HTEo$^-$s express approximately 10% of the maximal expression seen in the ΣCFTE29o$^-$ cells under basal conditions) (figure 46). To assess whether endogenous ligands for PPARγ affect the pattern of expression of this receptor, confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 6 hours and subsequently stimulated with increasing concentrations of 15-$dPGJ_2$ (0.1 – 10 μM), at 37°C, for 24 hours. No change in expression of PPARγ could be detected in response to stimulation with high concentrations of 15-$dPGJ_2$ (figure 47).

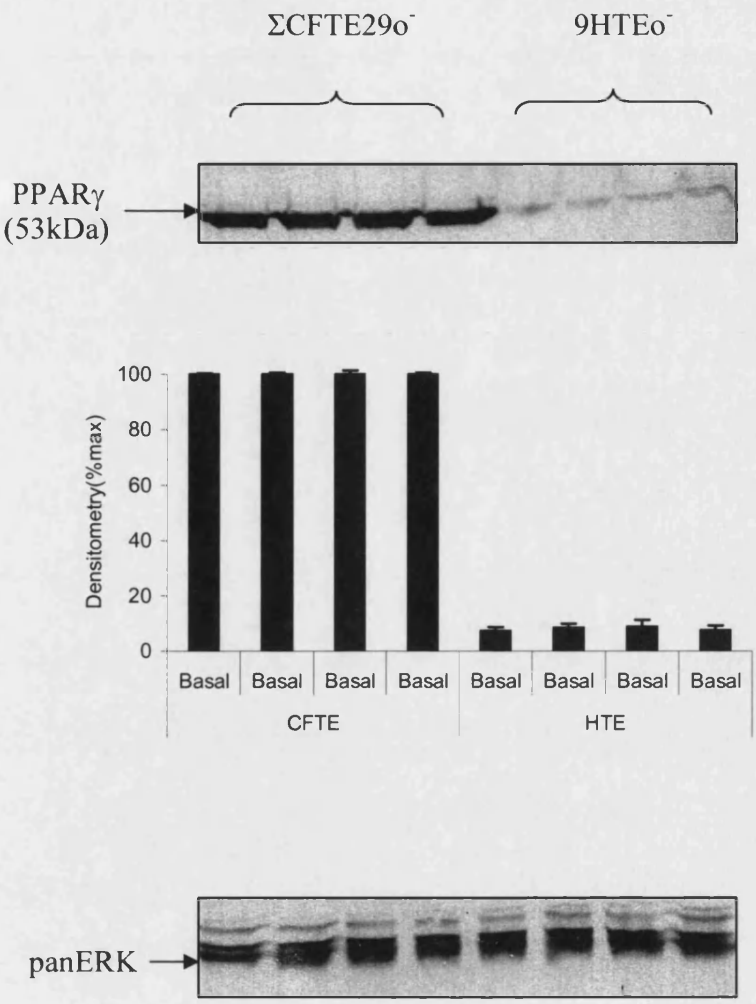



**Figure 46 Expression of PPARγ in ΣCFTE29o⁻ and 9HTEo⁻ cells**

Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to harvest. The top panel is the immunoblot probed with a specific antibody against PPARγ. Lanes 1 – 4 are unstimulated ΣCFTE29o⁻ samples from 4 separate experiments. Lanes 5 – 8 are unstimulated 9HTEo⁻ samples from 4 separate experiments. This immunoblot is representative of at least 3 other experiments. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 3 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

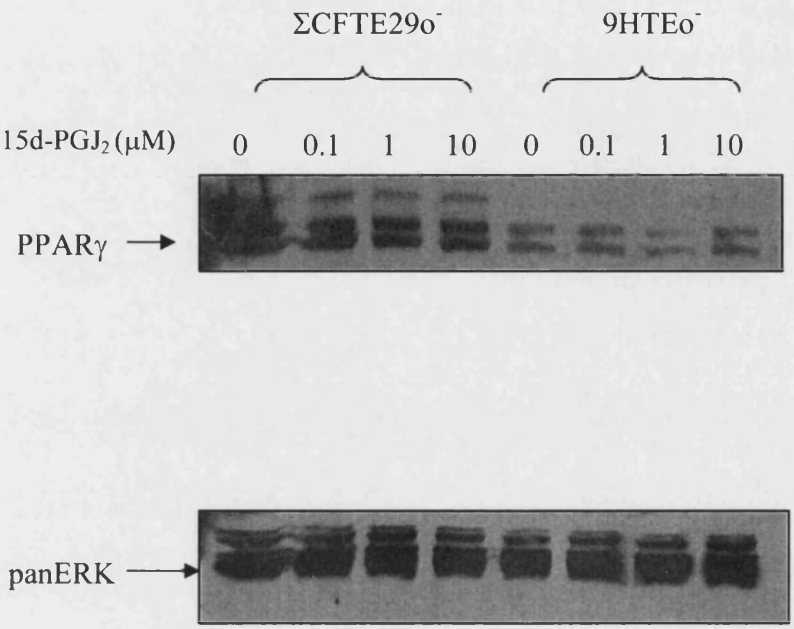



**Figure 47 Effect of 15d-$PGJ_2$ on expression of PPARγ ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of increasing concentrations of 15d-$PGJ_2$ (0.1 – 10 μM) on expression of PPARγ ΣCFTE29o⁻ and 9HTEo⁻ cells over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 4 hours prior to harvest. The top panel is the immunoblot probed with a specific antibody against PPARγ. This immunoblot is representative of 1 other experiment. The lower panel shows the membrane probed with an antibody against pan ERK to demonstrate equal loading.

### 5.2.2 The effect of PPARγ ligands on COX-2 protein expression and $PGE_2$ productoion in 9HTEo$^-$ (1) and ΣCFTE29o$^-$ (1) cells

Confluent monolayers of 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cells were serum starved for 6 hours and subsequently stimulated with vehicle or increasing concentrations of endogenous PPAR ligand, 15d-$PGJ_2$ (3nM - 3 μM), and synthetic PPARγ ligands troglitazone (3 nM - 3 μM) and GW2531 (3 nM - 3 μM) at 37°C for 24 hours. COX-2 protein expression was determined by immunoblot analysis, as described in the materials and methods section.

Initial investigation revealed that stimulation of ΣCFTE29o$^-$(1) cells with 15d-$PGJ_2$ (3nM - 3 μM) (figure 48A) and PPARγ ligand GW2531 (3 nM - 3 μM) (figure 48B) resulted in complete inhibition of COX-2 protein expression. Additionally, troglitazone (3 nM - 3 μM) reduced COX-2 expression in this cell line (figure 48C). These ligands appeared to have no effect upon COX-2 protein expression in 9HTEo$^-$(1) cells (figures 48 A,B, and C). $PGE_2$ ELISAs were performed, as detailed in the materials and methods section, on supernatants collected from 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cells incubated at 37°C overnight in the presence of vehicle, 15-d$PGJ_2$ (10 μM), or Troglitazone (10 μM). These Elisas revealed that while Troglitazone had little effect upon $PGE_2$ production by ΣCFTE29o$^-$(1) cells, 15-d$PGJ_2$ reduces $PGE_2$ generation by approximately 60% (figure 49). Once again, these ligands appeared to have no effect on 9HTEo$^-$(1) cells (figure 49).

As previously mentioned, because of problems with the growth of these cells a new batch of tracheal epithelial cells were ordered from the original source. These new cells, the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines were used for the rest of this study. The data obtained from this new batch of cells differs from those seen with the original 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines. As the new batch have been handled by the same person from arrival and their growth and data obtained consistent, this data from the 9HTEo$^-$ and ΣCFTE29o$^-$ cells are taken to be accurate. Therefore the 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines were put aside and the 9HTEo$^-$ and ΣCFTE29o$^-$ cells used throughout the rest of this study.

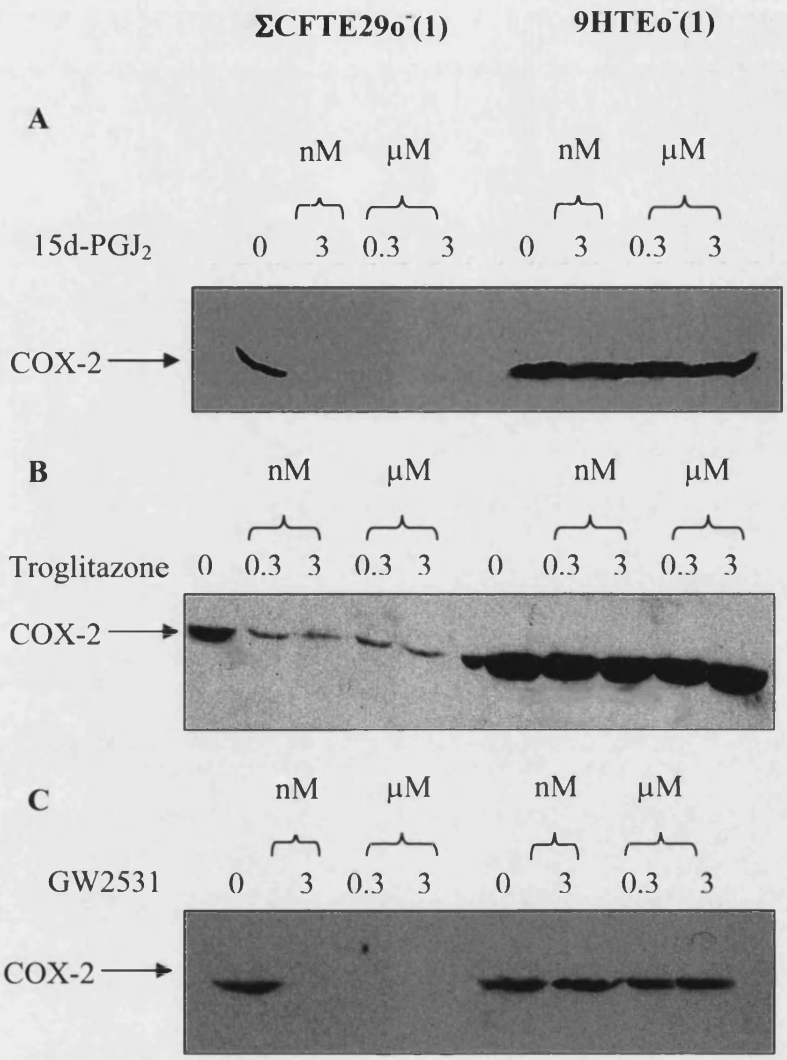



**Figure 48. Expression of COX-2 in *original batch* ΣCFTE29o⁻ (1) and *original batch* 9HTEo⁻ (1) cell lines, effect of PPARγ ligands**

A) Effect of exogenous application of increasing concentrations of 15d-$PGJ_2$ (3nM – 3μM), over 24 hours, on the ΣCFTE29o⁻(1) and 9HTEo⁻(1) cell lines. B) Effect of exogenous application of increasing concentrations of Troglitazone (3nM – 3μM)), over 24 hours on the ΣCFTE29o⁻(1) and 9HTEo⁻ cell lines. C) Effect of exogenous application of increasing concentrations of GW2351e (3nM – 3μM)), over 24 hours on the ΣCFTE29o⁻(1) and 9HTEo⁻ cell lines. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. These immunoblots are representative of 2 other experiments.

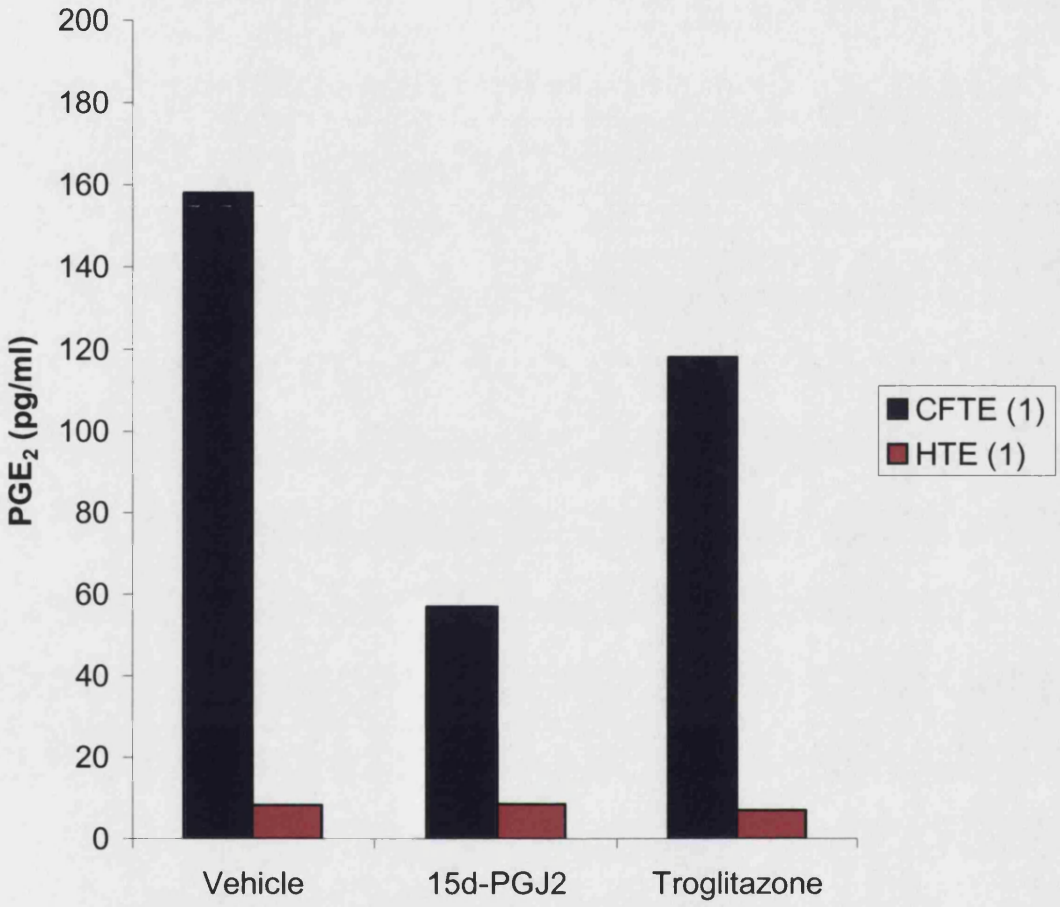



**Figure 49 $PGE_2$ production by *original batch* ΣCFTE29o⁻ (1) and *original batch* 9HTEo⁻ (1) cell lines, effect of PPARγ ligands**

The cells were serum starved for 6 hours prior to incubation with vehicle, 15-d$PGJ_2$ (10 μM) or Troglitazone (10 μM). Basal is the amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean for 2 experiments carried out in duplicate.

### 5.2.3 The effect of PPARγ ligands on COX-2 protein expression in 9HTEo$^-$ and ΣCFTE29o$^-$ cells

Confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 6 hours and subsequently stimulated with vehicle or increasing concentrations of endogenous PPAR ligand, 15d-$PGJ_2$ (1pM - 10 μM), synthetic PPARγ ligand troglitazone (1pM - 10 μM), and PPARγ antagonist BADGE (10pM - 10 μM) at 37°C for 24 hours. COX-2 protein expression was determined by immunoblot analysis, as described in the materials and methods section.

Increasing concentrations of 15-d$PGJ_2$ had no effect upon basal COX-2 expression in either cell line (figure 50 A and B). Similarly troglitazone did not appear to have any effect upon COX-2 protein expression in either 9HTEo$^-$ or ΣCFTE29o$^-$ cells, at any of the concentrations used in this study (figure 51 A and B). Additionally, PPARγ antagonist BADGE failed to induce a change in the expression of COX-2 by the airway epithelial cell lines (figure 52 A and B). To be certain that a change in COX-2 protein expression, induced by stimulation with PPARγ ligands, hadn't occurred at an earlier time point, confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 6 hours and subsequently incubated for 2 and 6 hours, at 37°C, with either vehicle or 10 μM $PGJ_2$. No change, in COX-2 expression, from basal could be observed in either cell line at either 2 or 6 hours in response to 10 μM 15d-$PGJ_2$ (figure 53).

### 5.2.4 The effect of PPARγ ligands on PGES protein expression in the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines

In order to investigate whether PPARγ ligands exert any effect on cPGES or mPGES protein expression either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell line, confluent monolayers of cells were serum starved for 6 hours and subsequently stimulated with vehicle or increasing concentrations of endogenous PPAR ligand, 15d-$PGJ_2$ (10pM -10 μM), synthetic PPARγ ligand troglitazone (10pM -10 μM), and PPARγ antagonist BADGE

(10pM - 10 μM) at 37°C for 24 hours and CPGES and mPGES protein expression determined by immunoblot analysis as described in the materials and methods section. A549 cells were used as a positive control for mPGES expression.

15d-$PGJ_2$ (10pM – 10 μM) had no discernable effect on either cPGES expression (figure 54 A) or mPGES expression (figure 44 B) in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines. Similarly, stimulation of the cells with troglitazone (10pM – 10 μM) did not alter basal cPGES (figure 55 A) or mPGES (figure 55 B) expression at the 24 hour time point. Incubation of the cell with PPARγ antagonist BADGE also failed to elicit any change in basal cPGES expression by either airway epithelial cell line (figure 56).

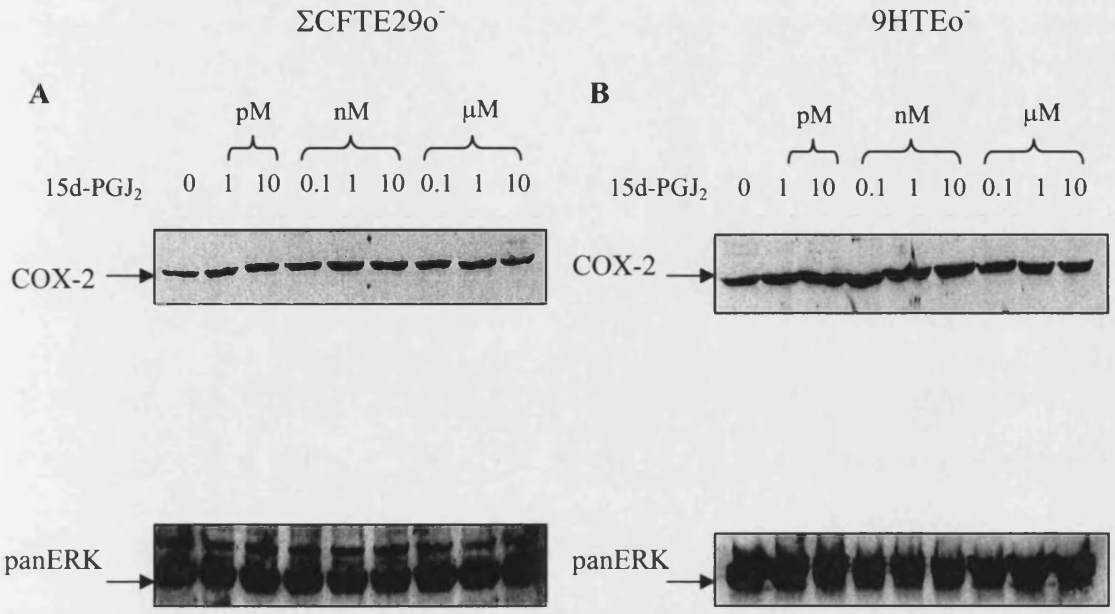



**Figure 50 Effect of 15d-$PGJ_2$ on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of 15d-$PGJ_2$ (1pM – 10μM), over 24 hours, on the ΣCFTE29o⁻ cell line. B) Effect of exogenous application of increasing concentrations of 15d-$PGJ_2$ (1pM – 10μM)), over 24 hours on the 9HTEo⁻ cell line. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. These immunoblots are representative of 4 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

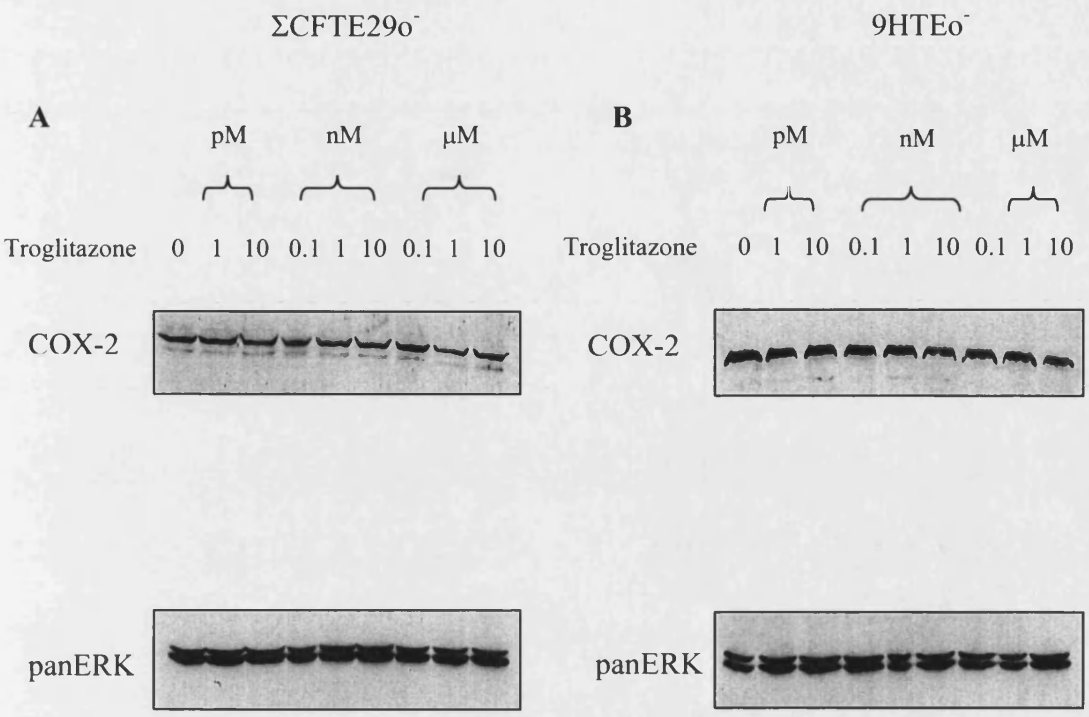



**Figure 51 Effect of troglitazone on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of troglitazone (1pM – 10μM), over 24 hours, on the ΣCFTE29o⁻ cell line. B) Effect of exogenous application of increasing concentrations of troglitazone (1pM – 10μM)), over 24 hours on the 9HTEo⁻ cell line. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. These immunoblots are representative of 3 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

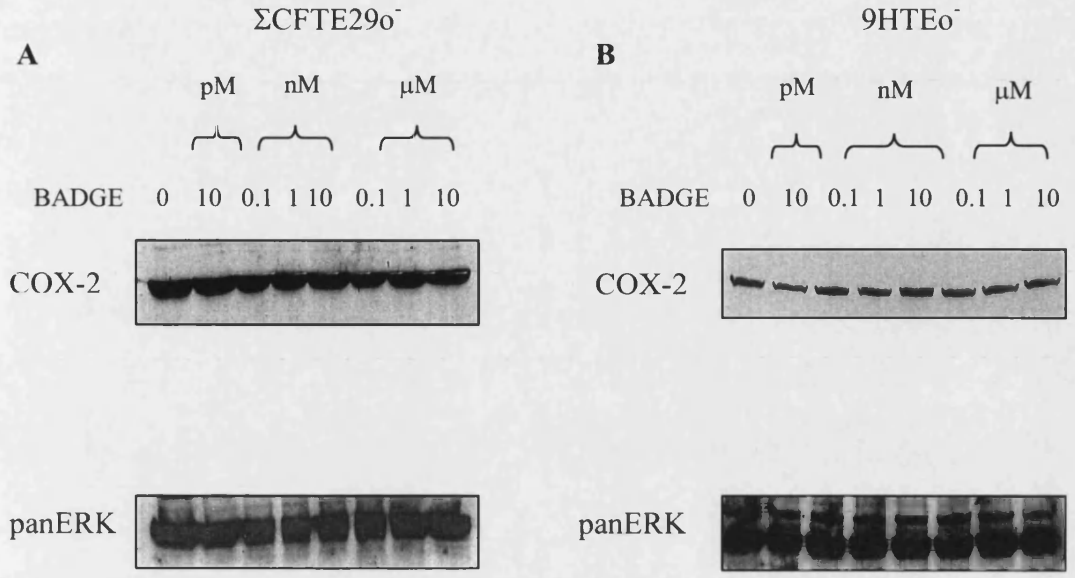



**Figure 52 Effect of BADGE on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of BADGE (10pM – 10μM), over 24 hours, on the ΣCFTE29o⁻ cell line. B) Effect of exogenous application of increasing concentrations of BADGE (10pM – 10μM)), over 24 hours on the 9HTEo⁻ cell line. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. These immunoblots are representative of 4 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

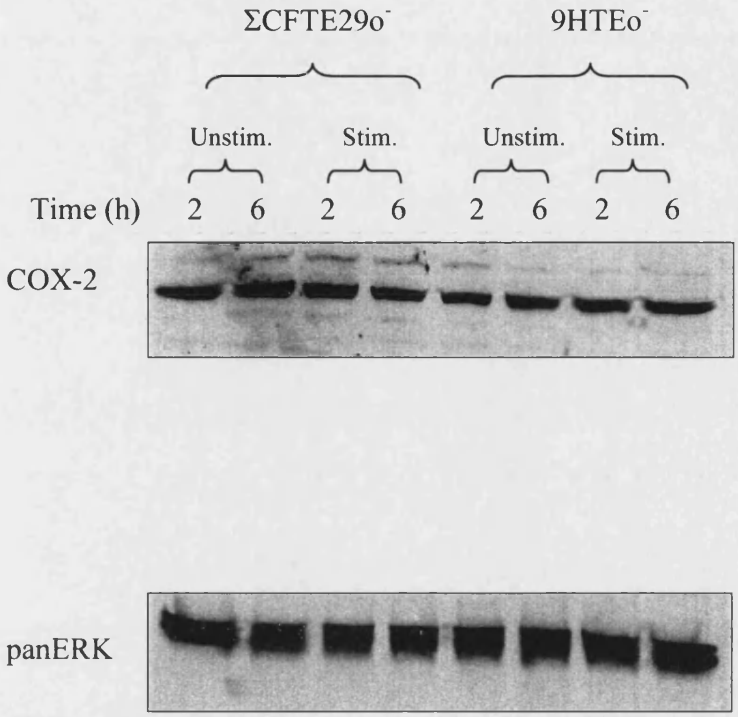



**Figure 53 Effect of 15d-$PGJ_2$ on expression of COX-2 in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

Two and six hour time points post stimulation with either vehicle (Unstim.) or 15d-$PGJ_2$ (10μM) (Stim.). Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against COX-2. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

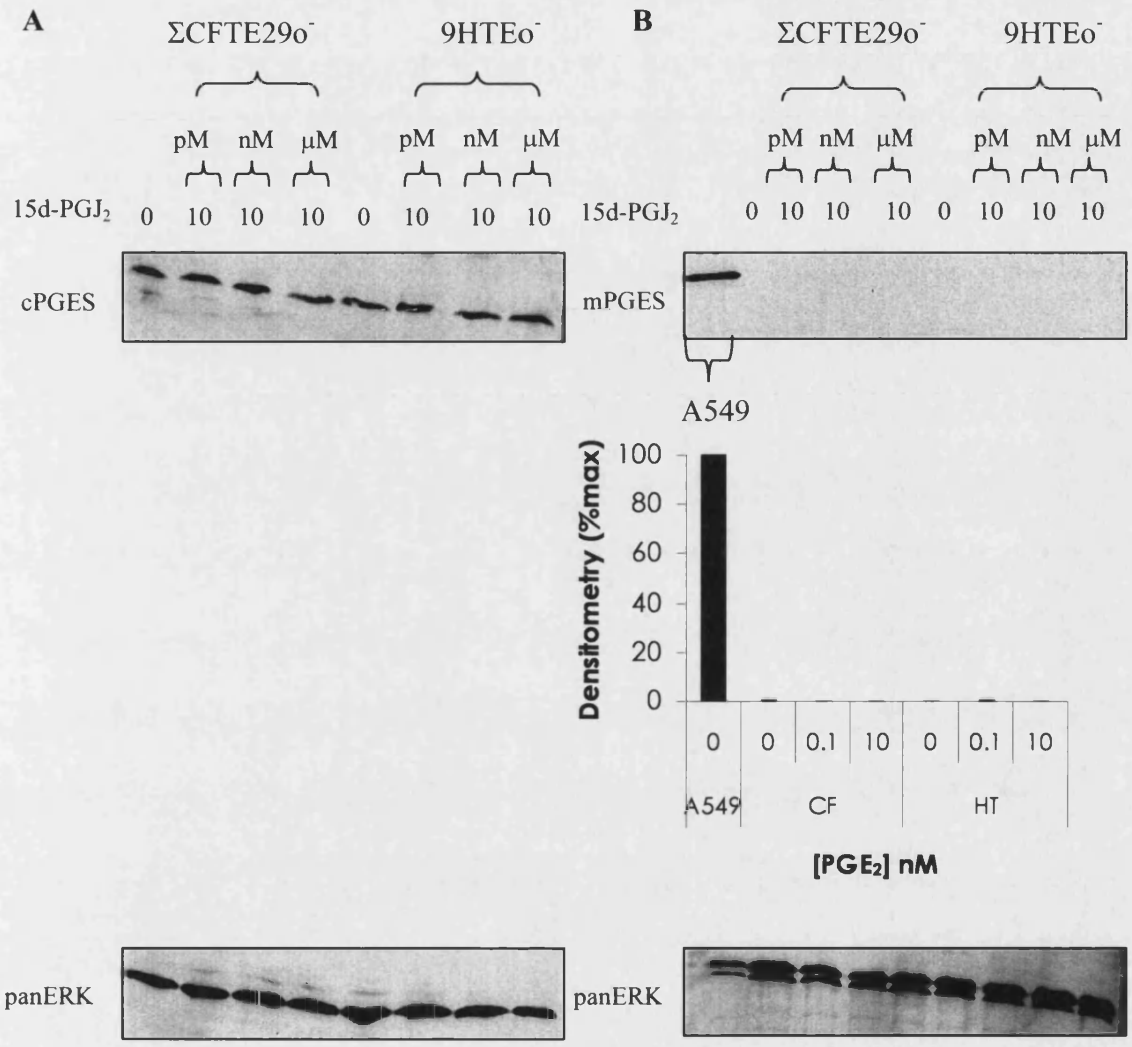



**Figure 54 Expression of cPGES or mPGES protein in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of 15d-$PGJ_2$ (10pM - 10 nM), over 24 hours on cPGES expression B) Expression of mPGES protein in A549, ΣCFTE29o⁻ and 9HTEo⁻ cell lines – Effect of exogenous application of increasing concentrations of 15-d$PGJ_2$ (10pM - 10 nM), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against A) cPGES and B) mPGES, these immunoblots are each representative of one other. The middle panel is the densitometry of the immunoblot (each bar is the mean of 2 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

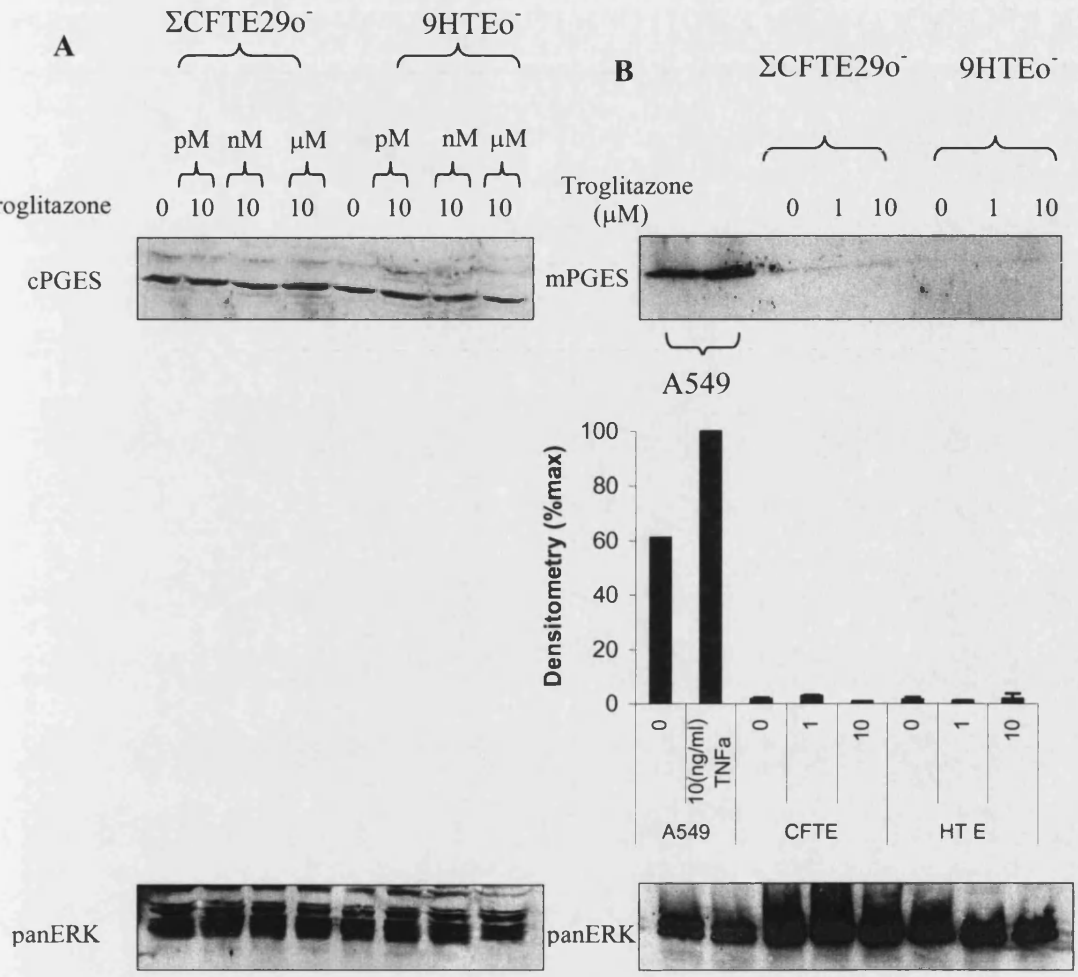



**Figure 55 Expression of cPGES or mPGES protein in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

A) Effect of exogenous application of increasing concentrations of 15d-$PGJ_2$ (10pM - 10 nM), over 24 hours, on cPGES expression B) Expression of mPGES protein in A549, ΣCFTE29o⁻ and 9HTEo⁻ cell lines – Effect of exogenous application of increasing concentrations of 15-d$PGJ_2$ (1 - 10 nM), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against A) cPGES and B) mPGES, these immunoblots are each representative of one other. The middle panel is the densitometry of the immunoblot (each bar is the mean of 2 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

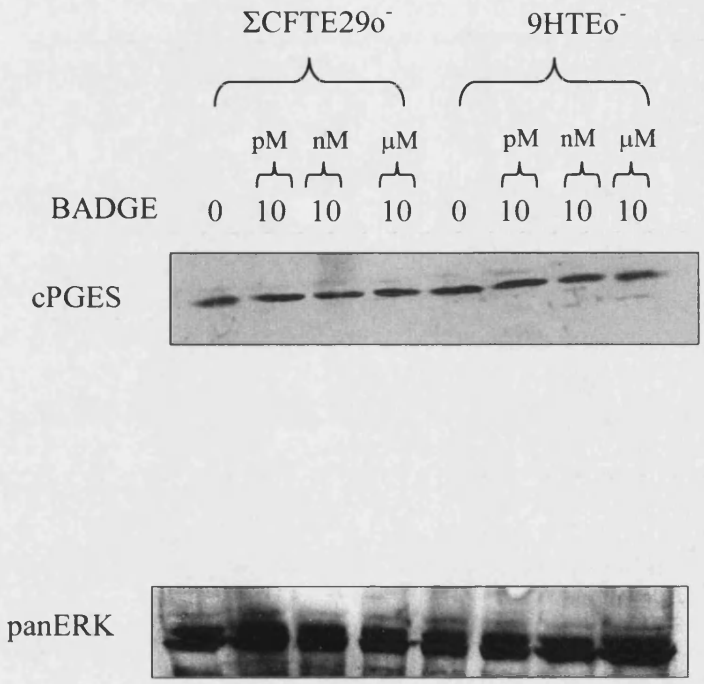



**Figure 56 Effect of BADGE on expression of cPGES protein in ΣCFTE29o⁻ and 9HTEo⁻ cell lines**

Effect of exogenous application of increasing concentrations of BADGE (10pM - 10 nM), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against cPGES. This immunoblot is representative of one other. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

### 5.2.5 The effect of PPARγ ligands on $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells

In order to determine the effect of PPARγ ligands on $PGE_2$ production by the ΣCFTE29o⁻ and 9HTEo⁻ cell lines, monolayers of cells were grown until confluent and serum starved for 6 hours prior to incubation, at 37°C, with either vehicle or 15-$dPGJ_2$ (10 μM) for 2, 6, and 24 hours. Additionally, the effect of PPARγ ligands on TNFα (10 ng/ml) induced $PGE_2$ generation, was investigated by pre-incubating the cells with 15-$dPGJ_2$ (10 μM) or troglitazone (10 μM) for 30 minutes prior to 24 hour stimulation with TNFα. Supernatants were collected and $PGE_2$ determined by $PGE_2$ ELISA as described in the materials and methods section.

In the ΣCFTE29o⁻ cell line, 15-$dPGJ_2$ (10 μM) appeared to induce $PGE_2$ production at the 2, 6 and 24 hour time points ($n$ =2). 15-$dPGJ_2$ (10 μM) also induced an increase in $PGE_2$ generation by the 9HTEo⁻ cell line at the 2, 6, and 24 hour time points ($n$ = 2) (figure 57). Troglitazone (10 μM) appeared to inhibit $PGE_2$ production by the both cell lines. Additionally, troglitazone inhibited TNFα (10 ng/ml) induced $PGE_2$ generation by ΣCFTE29o⁻ cells ($n$ =2) (figure 58). Pre-incubation of ΣCFTE29o⁻ and 9HTEo⁻ cells with 15-$dPGJ_2$ (10 μM) appeared to reduce TNFα induced $PGE_2$ production (figure 58), whether this reduction is significant or not requires further investigation. In an attempt to determine whether the effect of 15-$dPGJ_2$ on TNFα induced $PGE_2$ generation could be an effect of 15-$dPGJ_2$s actions on NFκB, IL-8 ELISAs were carried out on supernatants from the same cell cultures as used for the $PGE_2$ ELISA. 15d-$PGJ_2$ did not appear to have any effect upon basal IL-8 production or TNFα stimulated IL-8 generation by ΣCFTE29o⁻ and 9HTEo⁻ cells (figure 59).

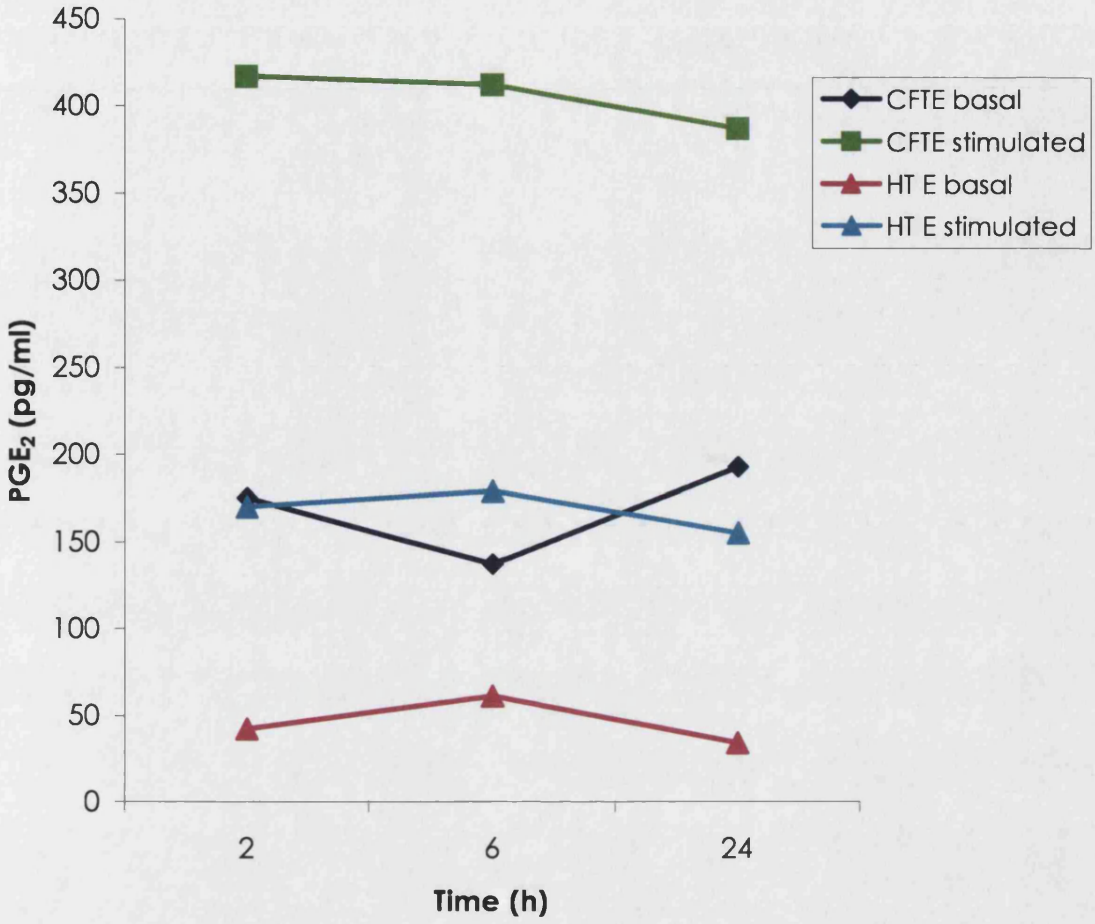



**Figure 57 Time course of $PGE_2$ production by ΣCFTE29o⁻ and 9HTEo⁻ cells after incubation with vehicle or 15-$dPGJ_2$ (10 μM)**

The cells were serum starved for 6 hours prior to treatment. Basal is the amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean for 2 experiments carried out in duplicate.

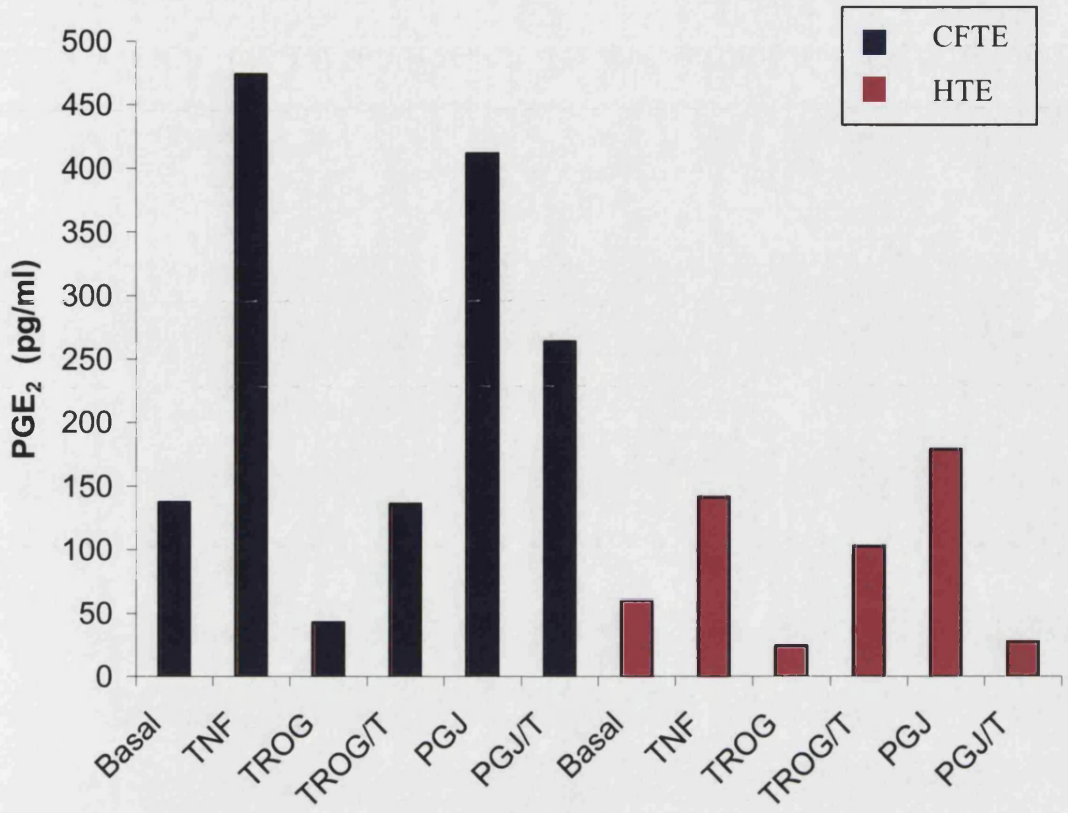



**Figure 58 Effect of TNFα, 15d-$PGJ_2$ and troglitazone on $PGE_2$ production at the 24 hour time point by ΣCFTE29o⁻ and 9HTEo⁻ cells**

The cells were serum starved for 6 hours prior to incubation with vehicle, TNFα (10ng/ml), or 15-d$PGJ_2$ (10 μM) either individually or in combination or Troglitazone (T) (10 μM) either alone or in combination with TNFα (10ng/ml). Basal is the amount of $PGE_2$ produced in the presence of vehicle alone. Each point is the mean for 2 experiments carried out in duplicate.

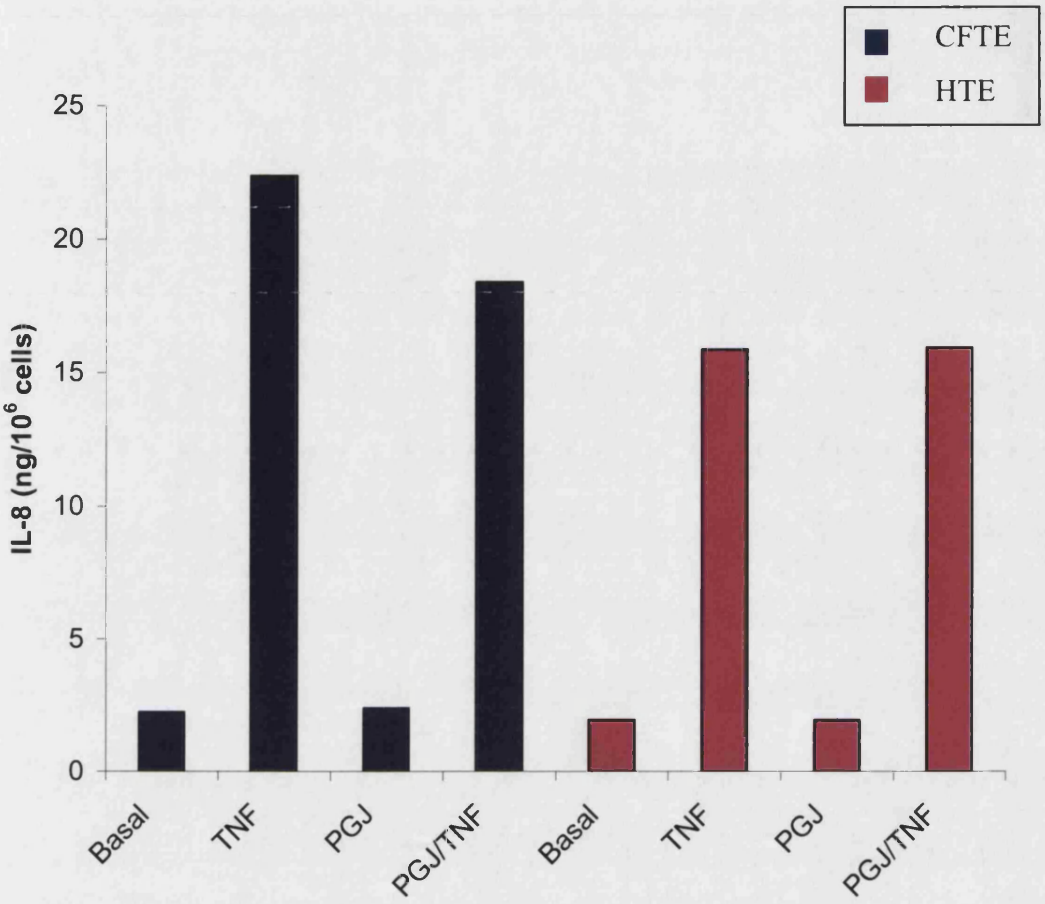



**Figure 59 Effect of TNFα and 15d-$PGJ_2$ on IL-8 production at the 24 hour time point by ΣCFTE29o⁻ and 9HTEo⁻ cells**

The cells were serum starved for 6 hours prior to treatment with vehicle, TNFα (10ng/ml), or 15-$dPGJ_2$ (10 μM) either individually or in combination. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean for 2 experiments carried out in duplicate.

### 5.2.6 The effect of PPARγ ligands on the expression of PGDH in ΣCFTE29o⁻ and 9HTEo⁻ cells

Confluent monolayers of 9HTEo⁻ and ΣCFTE29o⁻ cells were serum starved for 6 hours and subsequently stimulated with vehicle or increasing concentrations of endogenous PPAR ligand, 15d-$PGJ_2$ (1 - 10 μM), synthetic PPARγ ligand troglitazone (1 - 10 μM), and PPARγ antagonist BADGE (1 - 10 μM) at 37°C for 24 hours. PGDH protein expression was determined by immunoblot analysis as described in the materials and methods section.

PGDH protein expression was not affected by incubation with 15d-$PGJ_2$ (1-10 μM) at the 24hour time point in either the 9HTEo⁻ or ΣCFTE29o⁻ cell line (figure 60 A). Troglitazone (1 – 10 μM) did not induce any detectable change in the expression of PGDH in either cell line (figure 60 B). Furthermore, PPARγ antagonist BADGE (1 - 10 μM) failed to induce an effect on PGDH protein expression in either 9HTEo⁻ or ΣCFTE29o⁻ cells (figure 60 C).

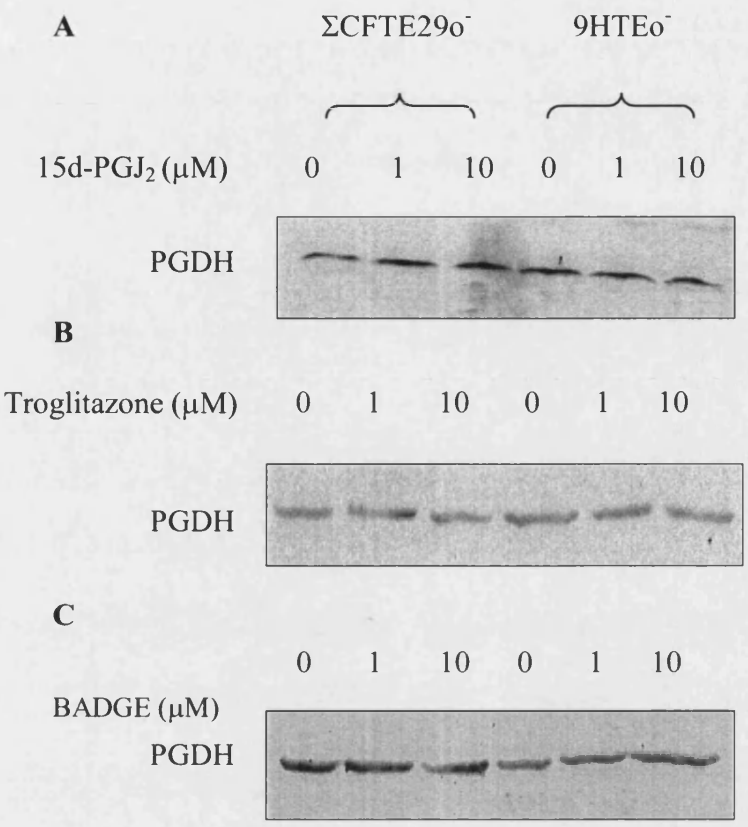



**Figure 60 Expression of PGDH protein ΣCFTE29o⁻ and 9HTEo⁻ cells**

Effect of exogenous application of A) 15d-$PGJ_2$ (0 ,1, and 10 nM) B) Troglitazone (0.1, and 10 nM) and C) BADGE (0 ,1, and 10 nM), over 24 hours. Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to treatment. The three panels are immunoblots probed with a specific antibody against PGDH.

## 5.3 Discussion

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. The PPARγ subtype is reported to be expressed in high levels in the airway epithelium (Michael *et al.,* 1997; Wang *et al.,* 2001). A number of studies have demonstrated that both natural and synthetic ligands of PPARγ possess anti-inflammatory properties, attenuating the release of macrophage-derived cytokines such as IL-1 and TNFα (Jiang *et al.,* 1998; Ricote *et al.,*1998; Welch *et al.,* 2003) and regulating expression of COX-2 and inducible nitric oxide synthase (Colville-Nash *et al.,* 1998). Wang *et al.,* 2001 report that activation of PPARγ in airway epithelial cell lines dramatically inhibits cytokine-induced expression of inflammatory mediators by these cells and suggest that PPARγ may act as a negative immunomodulator in the airways. This is supported by the findings of Patel *et al.,* 2003 who report that PPARγ ligands suppress cell growth, inhibit apoptosis, and inhibit GM-CSF release from HASM cells. The apparent ability of PPARγ ligands to down-regulate inflammatory pathways makes this receptor isoform a potential target for therapies aimed at disorder in which the inflammatory response is elevated or dysregulated. Studies into the effect of PPARγ ligands have revealed that the effect of this receptor and its ligands on COX-2 expression are tissue specific. Elucidation of the tissue specific effects of PPARγ in inflammation is necessary in the development of new approaches to therapy for chronic inflammatory diseases based upon the effects of PPARγ ligands. Additionally, a number of studies report that the anti-inflammatory actions of PPARγ ligands are not only attributable to activation of PPARγ, but to modulation of transcription factors including NF-κB (Castrillo *et al.,* 2000; Straus *et al.,*2000). In diseases such as CF where activation of NF-κB is thought to be exaggerated resulting in increased inflammatory mediator production, PPARγ ligands may prove effective in reducing inflammation and airway damage. In light of these findings, this study investigated the expression of PPARγ and the effect of PPARγ ligands on $PGE_2$ production in 9HTEo$^-$ and ΣCFTE29o$^-$ cells, human tracheal epithelial cell lines with a non-CF and CF phenotype respectively.

Both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines constitutively expressed PPARγ, however, expression of this receptor by ΣCFTE29o$^-$ cells was markedly higher than expression in 9HTEo$^-$ cells. As these cell lines are not a genetically matched pair, it is difficult to ascertain whether or not the differences observed in receptor expression between the CF and non-CF cells have any relevance. Attempts were made to assess relative PPARγ expression in a genetically matched pair of cell lines expressing a CF and non-CF phenotype, however, lack of reproducibility of data from these cells resulted in the data being disregarded. Incubation of the cell lines with high concentrations (μM range) of endogenous PPARγ ligand 15d-$PGJ_2$ did not produce any significant effect upon expression of PPARγ in either cell line.

Inoue *et al.,* 2000 report that COX-2 protein expression is regulated by a negative feedback loop mediated through PPARγ making possible a dynamic production of PG in the macrophage-like cell line U937. $PGD_2$ degradation product 15d-$PGJ_2$ is an endogenous high affinity ligand for PPARγ (Forman *et al.,* 1995; Kliewer *et al.,* 1995) *in vivo* levels of which are reportedly in the low pM range (Shibata *et al,* 2002; Bell-Parikh *et al.,* 2003). 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines were incubated with concentrations of 15d-$PGJ_2$ ranging from 1pM to 10μM for 24 hours. No change in COX-2 protein expression was be observed in either cell line in response to stimulation with this PG. Additionally, incubation of these cells with synthetic PPARγ ligand troglitazone or endogenous PPARγ antagonist BADGE at concentrations ranging from from 1pM to 10μM had no effect upon COX-2 expression. Furthermore, no significant change in cPGES or mPGES enzymes could be induced by the PPARγ ligands used in this study. These data suggest that modulation of COX-2 and PGES enzyme expression in the tracheal epithelium is not a valid target for PPARγ based therapeutic approaches.

Despite the inability of PPARγ ligands to affect COX-2, cPGES or mPGES protein expression in 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, $PGE_2$ ELISAs revealed that in response to stimulation with 10 μM 15d-$PGJ_2$, $PGE_2$ is increased in both cell lines at the

2, 6, and 24 hour time points. These data suggest that 15d-$PGJ_2$ is able to modulate the activity rather than the expression of $PGE_2$ synthesising enzymes. Additionally, the effects of 15d-$PGJ_2$ on TNFα induced $PGE_2$ production was assessed, in contrast with the finding that 15d-$PGJ_2$ alone enhances $PGE_2$ generation, 15d-$PGJ_2$ appears to inhibit TNFα induced $PGE_2$ production. However, further investigation is required to determine the significance of these results. These data pose interesting questions about the mechanisms behind the actions of 15d-$PGJ_2$ on basal $PGE_2$ generation versus its action upon $PGE_2$ generation stimulated by pro-inflammatory mediators. Unfortunately, this study does not provide evidence to explain the mechanism by which 15d-$PGJ_2$ exerts its effect upon $PGE_2$ synthesis. Interestingly, troglitazone appeared to inhibit basal $PGE_2$ production by both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines and to inhibit TNFα induced $PGE_2$ generation by ΣCFTE29o$^-$ cells. It is not possible to conclude that the effect of troglitazone on $PGE_2$ production is solely a product of PPARγ activation as, while Castrillo *et al.* (2000) suggest that 15d-$PGJ_2$ but not troglitzone is able to ligate to and modulate the NFκB signalling pathway, a number of studies have reported that thiazolidinediones troglitazone and rosiglitazone are able to inhibit NFκB activity (Ghanim *et al.*, 2001; Mohanty *et al.*, 2004). The mechanisms behind the conflicting effects of PPARγ ligands on $PGE_2$ production provide an interesting avenue for future research.

As previously mentioned, a number of studies report that the anti-inflammatory actions of PPARγ ligands are not only attributable to activation of PPARγ, but to modulation of transcription factors including NF-κB (Castrillo *et al.*, 2000; Straus *et al.*,2000). Concentrations of 15d-$PGJ_2$ required to ligate proteins in the NF-κB pathway *in vitro* range from 2.5 – 100 μM (Castrillo *et al.*, 2000; Narumiya and Fitzgerald, 2001). The concentration of 15d-$PGJ_2$ producing an effect upon $PGE_2$ production in this study was 10 μM and therefore within the concentration range capable of affecting the NF-κB pathway. In order to determine whether the effects of 15d-$PGJ_2$ on $PGE_2$ production could be a result of 15d-$PGJ_2$s actions upon NF-κB, IL-8 ELISAs were performed on the supernatants from cells stimulated with TNFα and 15d-$PGJ_2$ alone and from cells pre-incubated with 15d-$PGJ_2$ prior to TNFα stimulation. As IL-8 production is governed by the activity NF-κB (Reviewed in Hoffmann *et al.*, 2002), it is reasonable to

assume that if 15d-$PGJ_2$ is exerting a significant effect upon this transcription factor it should be reflected in IL-8 generation. No difference in basal IL-8 concentration or TNFα could be detected in response to 15d-$PGJ_2$. These data suggest that at the concentration and time point observed in this study, 15d-$PGJ_2$ does not significantly affect the activity of the transcription factor NF-kB in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines.

The increase in $PGE_2$ production by 15d-$PGJ_2$ could not be explained by changes in the expression of $PGE_2$ biosynthetic enzymes, thus the effect of PPARγ ligands on $PGE_2$ metabolising enzyme PGDH were investigated. Once again, no change in enzyme expression was observed in response to incubation with μM concentrations of 15d-$PGJ_2$, troglitazone, or BADGE.

PPARγ ligands have been demonstrated to negatively regulate inflammatory pathways in A549 cells (Wang *et al.,* 2000; Trifilieff *et al.,* 2003), HASM cells (Patel *et al.,* 2003), and bronchial epithelial cells (Hetzel *et al.,* 2003). Thus the inability of PPARγ ligands to significantly either positively or negatively regulate the expression of enzymes involved in the biosynthesis or catabolism of $PGE_2$ or IL-8 in either 9HTEo$^-$ or ΣCFTE29o$^-$ tracheal epithelial cell lines was surprising. However, the conflicting effects of PPARγ ligands on different tissues point to the effects of these ligands being highly tissue specific. The ability of 15d-$PGJ_2$ to up-regulate $PGE_2$ supports the findings of Inoue *et al,* 2000 in that 15d-$PGJ_2$ is able to modulate PG expression, however, in this study the modulation of PG production can not be attributed to changes in COX expression.

In summary, this study demonstrates that PPARγ is constitutively expressed in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$. Despite stimulation with a wide concentration range, PPARγ ligands do not affect protein expression of enzymes involved in the biosynthesis or catabolism of $PGE_2$. The mechanisms behind the ability of 15d-$PGJ_2$ to induce $PGE_2$ expression by the human tracheal epithelial cell lines

9HTEo$^-$ and ΣCFTE29o$^-$ could not be explained by the data gathered in this study, but the complex pro- and anti-inflammatory effects of $PGE_2$ raises the possibility that pharmacological use of 15d-$PGJ_2$ on the tracheal epithelium, could in some circumstances be detrimental to health.

The data gathered from the 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines, differs markedly from the data obtained from the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. The ability of PPARγ ligands to abrogate COX-2 in ΣCFTE29o$^-$(1) cells but not ΣCFTE29o$^-$ cells indicates that changes may occur in this cell line over time. The difference in data and problems with the growth of the older 9HTEo$^-$(1) and ΣCFTE29o$^-$(1) cell lines suggest that caution should be employed when using these cell lines to investigate the effect of PPARs on PG generation as age related changes to the cell lines appear to affect these pathways.

## 5.4 Summary of Results

- PPARγ is constitutively expressed in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines ΣCFTE29o$^-$ cells express higher levels of PPARγ than 9HTEo$^-$ cells
- PPARγ ligands 15d-$PGJ_2$, troglitazone, and BADGE do not affect basal COX-2 expression in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines
- 15d-$PGJ_2$ appears to increase $PGE_2$ production by both cell lines, but does not have an effect upon TNFα induced $PGE_2$ generation
- Troglitazone appears to inhibit basal $PGE_2$ production in both airway epithelial cell lines. Additionally, troglitazone did appear to reduce TNFα induced $PGE_2$ generation by ΣCFTE29o$^-$ cells
- IL-8 production was not affected by 15d-$PGJ_2$ in either 9HTEo$^-$ ΣCFTE29o$^-$ cells
- PGDH protein expression is not affected by PPARγ ligands 15d-$PGJ_2$, troglitazone, and BADGE

# Chapter 6: The role of prostaglandins in cytoprotection

## 6.1 Introduction

It has been reported that 15d-$PGJ_2$ can induce expression of HO-1, an enzyme with anti-oxidant activity in a number of cell lines (Colville-Nash *et al.,* 1998; Koppal *et al.,* 2000; Wayman *et al.,* 2002; Lee *et al.,* 2003). HO-1 is predominantly thought to be an inducible enzyme, expression of which is induced by pro-inflammatory cytokines, oxidative stress, and UV light. The aim of this study was to investigate the expression of HO-1 in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$ and to investigate the effect of PGs $PGE_2$ and 15d-$PGJ_2$ upon its expression.

## 6.2 Results

### 6.2.1 The Expression of HO-1 by 9HTEo$^-$ and ΣCFTE29o$^-$ cells

HO-1 protein expression by 9HTEo$^-$ and ΣCFTE29o$^-$ cells was investigated by immunoblot analysis as described in the materials and methods section. Confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 6 hours prior to incubation, at 37°C, with either vehicle or increasing concentrations of TNFα (0 – 10ng/ml), $PGE_2$ (0 – 10 μM), or 15d-$PGJ_2$ (0 – 10 μM).

HO-1 was expressed by both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines in the absence of cytokine stimulation. No difference in HO-1 expression could be detected between these cell lines. Stimulation of the cells with increasing concentrations of TNFα did not appear to effect basal expression of HO-1 in either airway epithelial cell line (figure 61). Incubation of 9HTEo$^-$ and ΣCFTE29o$^-$ cells with increasing concentrations of $PGE_2$ (0 – 10 μM) did not induce any detectable change in basal HO-1 expression by either cell line (figure 62). Stimulation of 9HTEo$^-$ with increasing concentrations of 15d-$PGJ_2$ (0 – 10 μM) did not have any effect upon HO-1 expression at the 24 hour time point (figure 63). However, incubation of ΣCFTE29o$^-$ cells with 10μM 15d-$PGJ_2$ induced a significant increase in HO-1 expression at the 24 hour time point, a small increase could be

observed in response to stimulation with 1μM 15d-$PGJ_2$, but no change in HO-1 expression was induced by 15d-$PGJ_2$ (0.1μM) (figure 63).

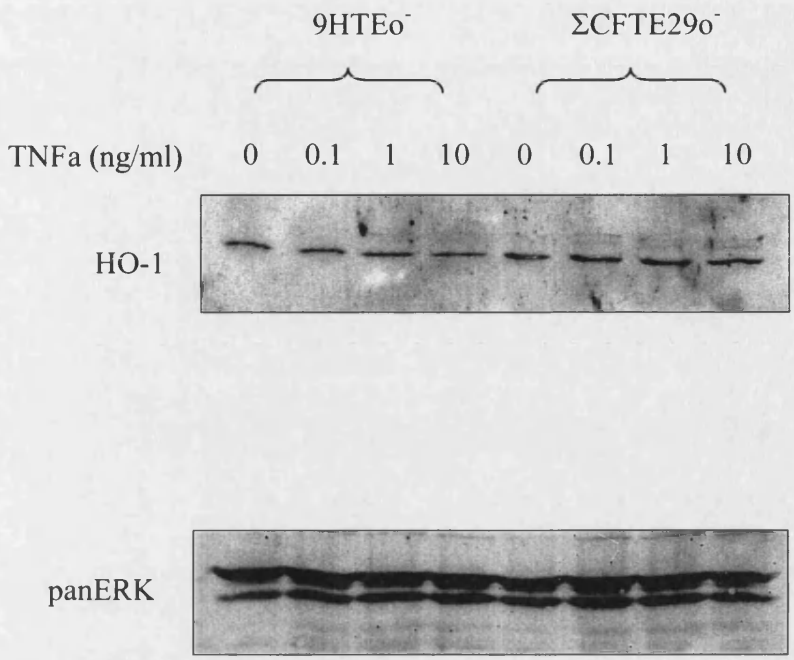



**Figure 61 Expression of HO-1 by 9HTEo⁻ and ΣCFTE29o⁻ cells – Effect of incubation with TNFα over 24 hours.**

Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to stimulation with TNFα (0.1 – 10 ng/ml) at 37°C. The top panel is the immunoblot probed with a specific antibody against HO-1. This immunoblot is representative of 2 other experiments. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

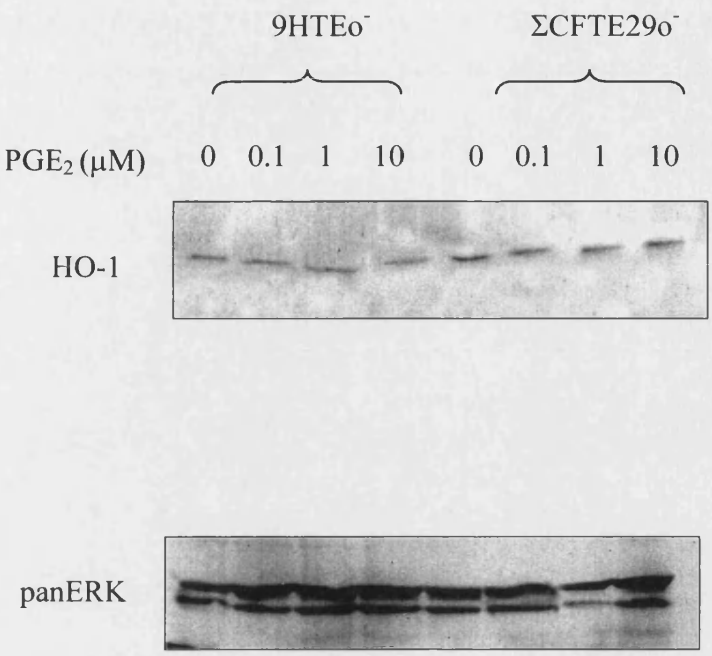



**Figure 62 Expression of HO-1 by 9HTEo⁻ and ΣCFTE29o⁻ cells – Effect of incubation with $PGE_2$ over 24 hours.**

Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to stimulation with $PGE_2$ (0.1 – 10 μM) at 37°C. The top panel is the immunoblot probed with a specific antibody against HO-1. This immunoblot is representative of 1 experiment. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

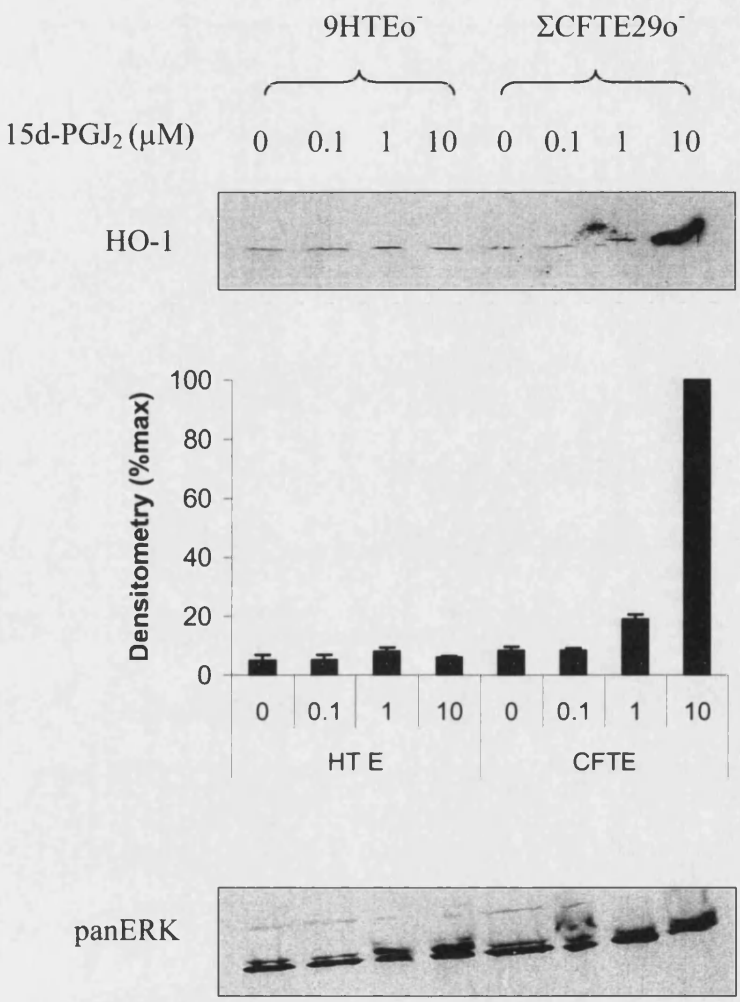



**Figure 63 Expression of HO-1 by 9HTEo⁻ and ΣCFTE29o⁻ cells – Effect of incubation with increasing concentrations of 15d-$PGJ_2$ (0 – 10 μM) over 24 hours.** Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to stimulation at 37°C. The top panel is the immunoblot probed with a specific antibody against HO-1. This immunoblot is representative of 2 other experiments. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 3 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading.

In order to determine whether 15d-$PGJ_2$ had an effect upon HO-1 expression at time points other that 24 hours, monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 6 hours and subsequently stimulated with 10μM 15d-$PGJ_2$ for 2, 6, 14 and 24 hours at 37°C.

In the ΣCFTE29o$^-$ cell line 10μM 15d-$PGJ_2$ did not appear to significantly affect HO-1 protein expression at either the 2 or 6 hour time points. However, by the 14 hour time point HO-1 expression had increased to approximately 25% of maximal expression. Maximal HO-1 expression was induced after 24 hour stimulation with 10μM 15d-$PGJ_2$ (figure 64 A). In 9HTEo$^-$ cells basal expression of HO-1 was unaffected by stimulation with 10μM 15d-$PGJ_2$ at the 2 hour time point. However, maximal expression of HO-1, for this cell line, was induced at the 6 hour time point in response to 10μM 15d-$PGJ_2$. HO-1 expression had returned to basal levels by the 14 hour time point and remained low at the 24 hour time point (figure 64 B). To investigate whether the increase in HO-1 expression, observed in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, in response to 15d-$PGJ_2$ was a result of activation of PPARγ monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 6 hours and subsequently stimulated with 10μM troglitazone for 2, 6, 14 and 24 hours at 37°C. No change in basal expression of HO-1 was detected in response to troglitazone in either ΣCFTE29o$^-$ cells (figure 65 A) or 9HTEo$^-$ cells (figure 65 B).

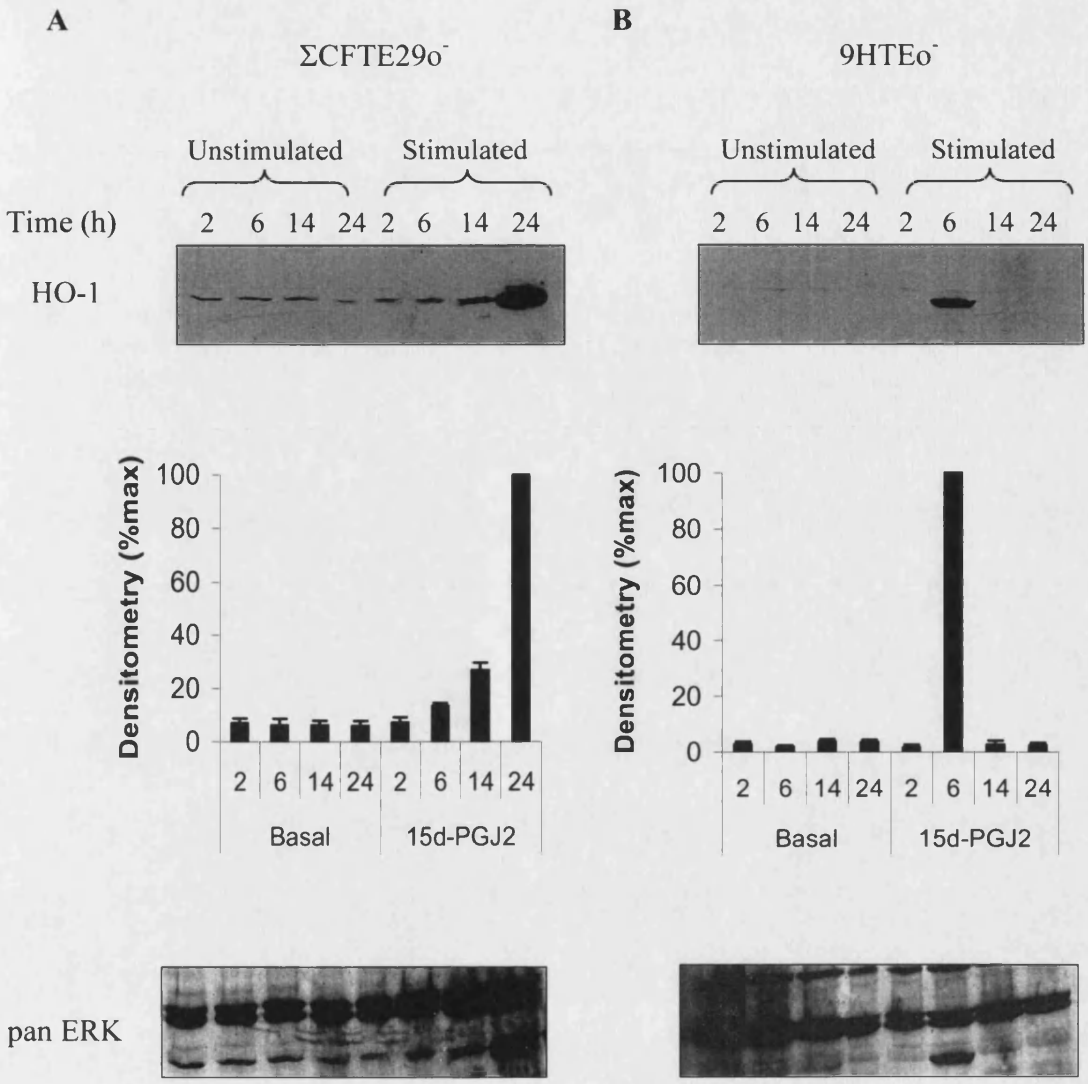



**Figure 64 Expression of HO-1 –Time course of effect of 15d-PGJ$_2$ (10 μM) in A) ΣCFTE29o⁻ cells and B) 9HTEo⁻ cells.**

Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to stimulation at 37°C. The top panel is the immunoblot probed with a specific antibody against HO-1. This immunoblot is representative of 2 other experiments. The middle panel is the densitometry of the immunoblot (each bar is the mean ± SEM of 3 experiments). The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

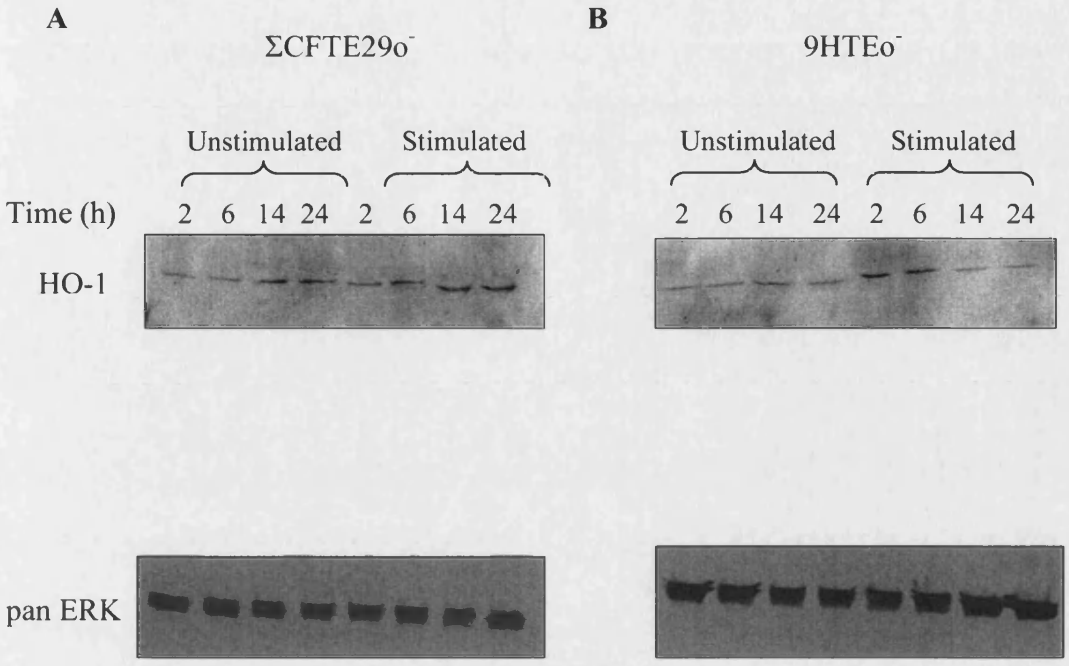



**Figure 65 Expression of HO-1 –Time course of effect of troglitazone (10 μM) in A) ΣCFTE29o⁻ cells and B) 9HTEo⁻ cells.**

Western blot analysis of whole cell lysates. Cells were serum starved for 6 hours prior to stimulation at 37°C. The top panel is the immunoblot probed with a specific antibody against HO-1. The lower panel shows membranes probed with an antibody against pan ERK to demonstrate equal loading

### 6.2.2 Effect of PGs and PPARγ ligands on the metabolic activity of 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines

Monolayers of cells were serum starved for 6 hours prior to stimulation with either vehicle, 15d-$PGJ_2$ (10μM), $PGE_2$ (10μM), or troglitazone (10μM) for 2, 6, 14, and 24 hours. MTT assays were subsequently conducted in order to investigate whether these ligands have an effect upon cellular metabolic activity in either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell line.

Incubation of ΣCFTE29o$^-$ cells with $PGE_2$ (10μM) did not significantly affect the basal metabolic activity of these cells at the 2, 6, 14, or 24 hour time points (figure 56). Similarly, 15d- $PGJ_2$ (10μM) did not alter the basal metabolic activity of ΣCFTE29o$^-$ cells at any of the time points observed in this study (figure 66). Furthermore, PPARγ ligand troglitazone (10 μM) did not appear to induce a change in the activity of this cell line (figure 66). Incubation of 9HTEo$^-$ cells with $PGE_2$ (10μM) over 2, 6, 14, and 24 hours, did not appear to have an effect on the basal metabolic activity of this cell line (figure 67). However, incubation of the 9HTEo$^-$ cell line with 15d- $PGJ_2$ (10μM) caused a significant decrease in the cells metabolic activity when compared to basal (from 88 ± 19% of max. absorbance (550nm) for basal to 41 ± 3 % of max absorbance (550 nm) for 15d- $PGJ_2$ stimulated cells $n = 3$ $p<0.05$). The metabolic activity of the 9HTEo$^-$ cell line had returned to levels comparable to basal by the 14 and 24 hour time points and 15d-$PGJ_2$ (10μM) was not determined to significantly change the basal metabolic activity of this cell line at these time point (figure 67). In contrast, troglitazone (10μM) had no effect on the basal metabolic activity of the 9HTEo$^-$ cell line at any of the time points observed in this study.

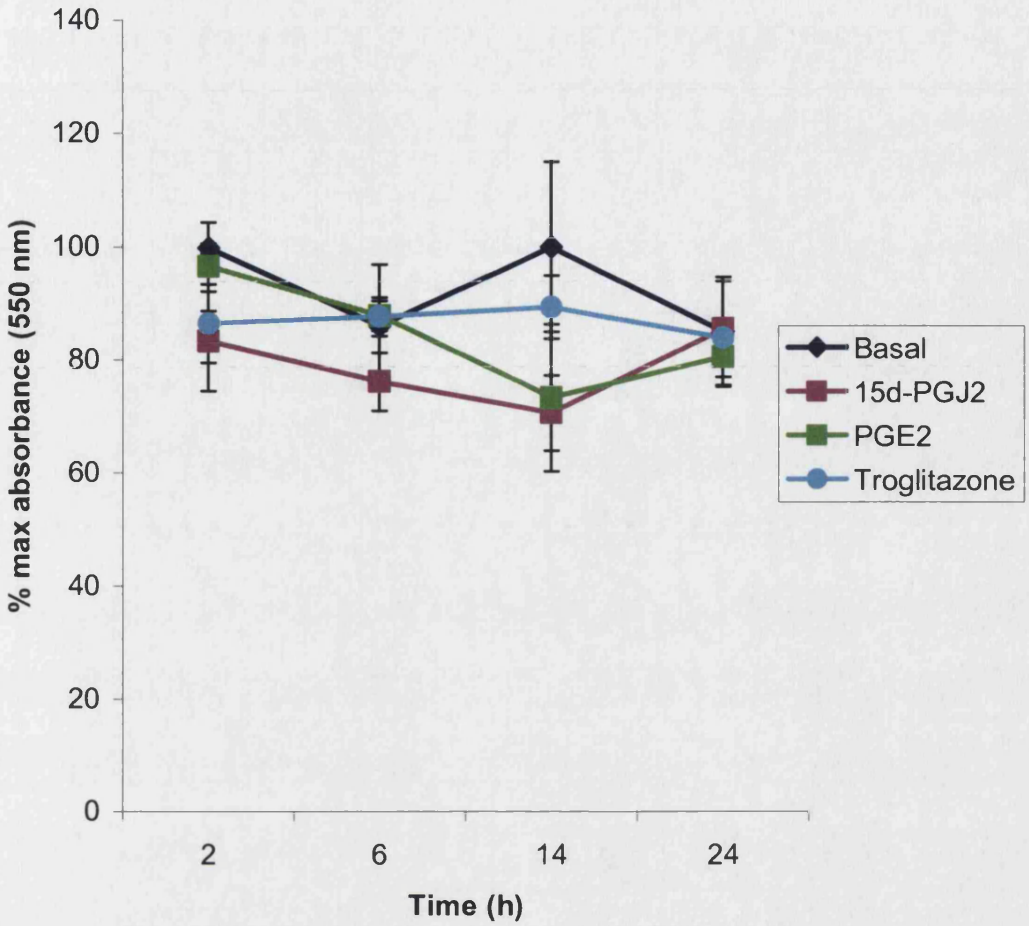



**Figure 66 Metabolic activity of ΣCFTE29o⁻ cells – Effect of incubation with vehicle, 15d-$PGJ_2$ (10 μM), $PGE_2$ (10 μM), or troglitazone (10 μM) over 2, 6, 14 and 24 hours.**

The cells were serum starved for 6 hours prior to analysis. Basal is the absorbance measured at a wavelength of 550 nm for cells stimulated with vehicle alone. Each bar is the mean ± SEM expressed as a percentage of the maximum absorbance for 3 experiments

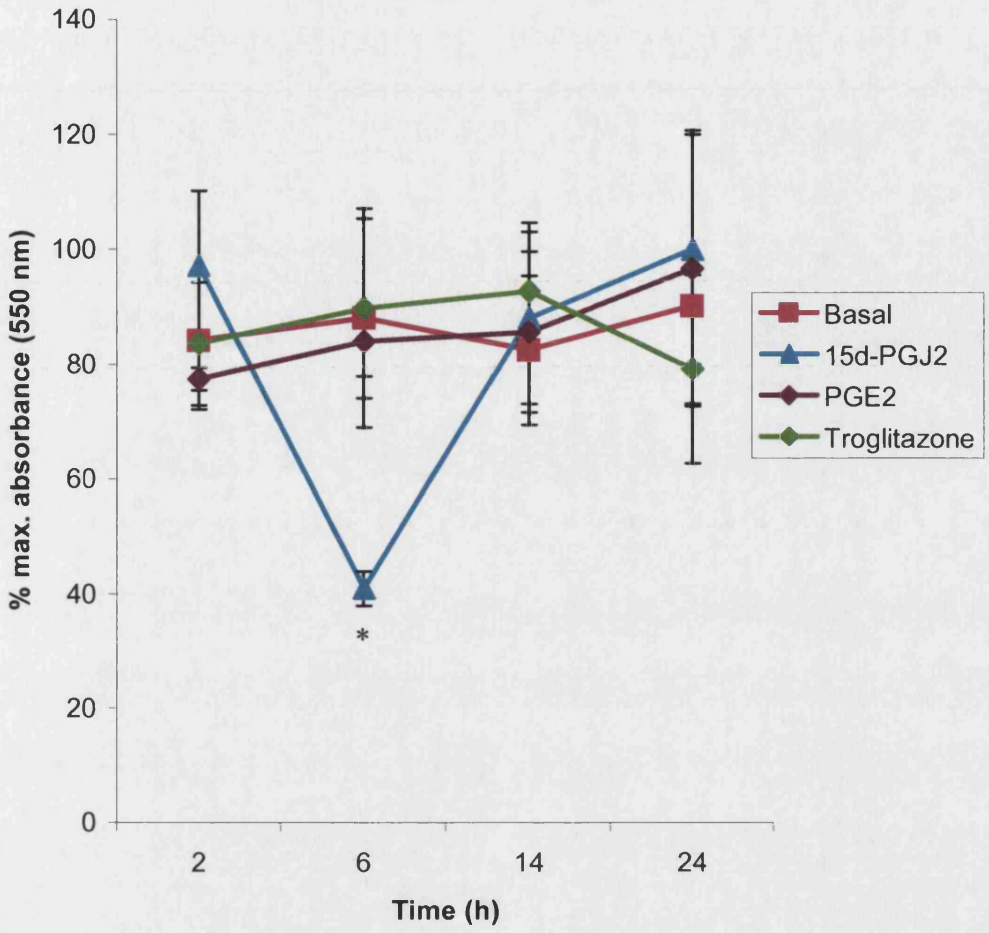



**Figure 67 Metabolic activity of 9HTEo⁻ cells – Effect of incubation with vehicle, 15d-PGJ$_2$ (10 µM), PGE$_2$ (10 µM), or troglitazone (10 µM) over 2, 6, 14 and 24 hours.**

The cells were serum starved for 6 hours prior to analysis. Basal is the absorbance measured at a wavelength of 550 nm for cells stimulated with vehicle alone. Each bar is the mean ± SEM expressed as a percentage of the maximum absorbance for 3 experiments (* $P<0.05$ compared to basal)

## 6.2 Discussion

Heme-oxygenase-1 (HO-1) is an inducible anti-oxidant enzyme that conveys protecion to oxidative cellular injury by the catabolism of pro-inflammatory heme to biliverdin and bilirubin, potent antioxidants; and carbon monoxide (CO), a neurotransmitter that has anti-inflammatory properties. Several studies report that HO-1 has anti-inflammatory properties. Li Volti *et al.,* 2003 report that HO-1 and its enzymatic products down-regulate the inflammatory response by repressing the induction of cytokines and chemokines. Additionally, Poss *et al.,* 1997 demonstrate that HO-1 deficient mice develop a chronic inflammatory state that increases with age. Furthermore, overexpression of HO-1 by gene transfer into rat lungs provided protection from hyperoxic injury (Otterbain *et al,* 1999). Paredi *et al.,* 1999 report an increase in exhaled CO levels, that may be of pathophysiological significance, in patients with cystic fibrosis. The authors of this study postulate that the source of this CO is HO-1. Donnelly and Barnes, 2001 report that HO-1 expression is inducible in airway epithelial cell lines and put forward the hypothesis that the airway epithelium may be the source of increased CO in airway diseases. It therefore seemed reasonable to investigate the expression of HO-1 in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$, cells of a CF and non-CF phenotype respectively. Additionally, prostaglandin 15d-$PGJ_2$ is considered to be an anti-inflammatory molecule with therapeutic properties production of which has been demonstrated to present in inflammatory exudates and is increased during the resolution phase of inflammation (Gilroy *et al,* 1999). Several studies indicate that 15d-$PGJ_2$ exerts its anti-inflammatory activity by inhibiting the expression of pro-inflammatory mediators (Ricote *et al.,* 1998; Jiang *et al.,* 1998; Cuzzocrea *et al.,* 2002). Recent studies have illustrated that 15d-$PGJ_2$ induces HO-1 expression in a variety of cells including activated microglial cells (Koppal *et al,* 2000), murine macrophages (Colville-Nash *et al.,* 1998; Lee *et al.,* 2003), cardiac myocytes (Wayman *et al.,* 2002), and human lymphocytes (Álvarez-Maqueda *et al.,* 2004). To date, the induction of HO-1 expression by 15d-$PGJ_2$ has not been reported in the human airway epithelium. In this study HO-1 protein expression has been investigated in 9HTEo$^-$ and ΣCFTE29o$^-$ cells and its induction by cytokines, $PGE_2$, 15d-$PGJ_2$ and troglitazone assessed.

Donnelly and Barnes, 2001 report that primary human airway epithelial cells express a low baseline of HO-1 mRNA under basal conditions; culture of these cells with a cytomix of TNFα, IL-1β, and IFNγ induces a fivefold increase in HO-1 expression at the 4 hour time point that decreases by the 12 hour time point. In this study low basal expression of HO-1 protein could be observed in both 9HTEo$^-$ and ΣCFTE29o$^-$ cells under basal conditions. Basal expression of HO-1 by the human tracheal epithelium may be induced by a continuous low level production of pro-inflammatory cytokines by these cells in response to a constant low-grade inflammatory response elicited by constant exposure to external stimuli. In this study expression of HO-1 could not be induced by pro-inflammatory cytokines at any time point between 2 and 24 hours. Furthermore, no difference in HO-1 expression could be observed between 9HTEo$^-$ and ΣCFTE29o$^-$ cells.

As previously stated, 15d-$PGJ_2$ has been reported to increase HO-1 expression in a number of tissues. As $PGE_2$ is the most abundant PG produced by the airway epithelium, and in this study expression of $PGE_2$ has been demonstrated to be elevated in ΣCFTE29o$^-$ cells compared to 9HTEo$^-$ cells, the effect of $PGE_2$ on HO-1 expression was investigated. However, no change in the expression of HO-1 could be detected in response to 24 hour stimulation with micro-molar concentrations of $PGE_2$. It is not possible to rule out the prospect of $PGE_2$ producing an effect upon HO-1 expression from these data, more physiologically relevant concentrations of $PGE_2$ could be used and earlier time points studied.

This study demonstrated that high concentrations of 15d-$PGJ_2$ (1 and 10 μM) induce HO-1 protein expression in the ΣCFTE29o$^-$ cells but not 9HTEo$^-$ cells. In order to elicit its an effect on HO-1 expression, concentrations of 15d-$PGJ_2$ substantially higher than those endogenously present at the sites of inflammation (pM - nM levels) ((Gilroy *et al.*, 1999) had to be used; this the effects of this PG on HO-1 expression in the airway epithelium could be of pharmacological rather than physiological relevance. Experiments constructed to investigate the time course of HO-1 induction by 15d-$PGJ_2$ revealed that HO-1 protein expression can be up-regulated in both cell lines. In the 9HTEo$^-$ cell line expression of this enzyme can be observed to be increased at the 6 hour

time point and to have reduced back to basal levels by the 14 hour time point. Induction of HO-1 expression is also observed to be induced by 15d-$PGJ_2$ at the 6 hour time point in ΣCFTE29o$^-$ cells, however, expression does not fall back to basal but continues to increase at the 14 and 24 hour time points. As 15d-$PGJ_2$ can act on cells through either PPARγ -dependent or –independent mechanisms, the same time course was constructed to determine whether synthetic PPARγ ligand troglitazone could also induce HO-1 protein expression. The inability of troglitazone to affect HO-1expression suggests that the induction of this enzymes expression by 15-d$PGJ_2$ may be PPARγ - independent.

The induction of HO-1 by 15d-$PGJ_2$ suggests that 15d-$PGJ_2$ may be inducing intracellular oxidative stress. In support of this, Chen *et al.,* 2002 report that, in a thyroid papillary cancer cell line 15d-PGJ2 caused cytotoxicity and increased the amount of intracellular reactive oxygen species (ROS) inducing apoptosis and/or inhibiting cell proliferation. Furthermore, Álvarez-Maqueda *et al.,* 2004 report that HO-1 induction in human lymphocytes is dependent on ROS produced via the xanthine/xanthine oxidase system and/or through Fenton reactions. Using MTT assays this study demonstrates that $PGE_2$, troglitazone and 15d-$PGJ_2$ have no effect on the basal metabolic activity of the ΣCFTE29o$^-$ cell line. This is despite 15d-$PGJ_2$ inducing significant levels of HO-1 protein. These data suggest that 15d-$PGJ_2$ is not cytotoxic to these cells. Similarly, $PGE_2$ and troglitzone did not significantly effect the basal metabolic activity of the 9HTEo$^-$ cell line. However, these experiments demonstrate that at the 6 hour time point 10 μM significantly reduces the metabolic activity of 9HTEo$^-$ cells; this occurs concomitantly with an increase in HO-1 expression. By the 14 hour time point both HO-1 expression and the basal metabolic activity of the 9HTEo$^-$ cell line have returned to levels not significantly different from time matched controls. These data indicate that 15d-$PGJ_2$ induces oxidative stress in the 9HTEo$^-$ cell line and that as a result HO-1 protein expression is induced. However, while the non-CF phenotype cell line can be observed to recover from the effects of stimulation with 15d-$PGJ_2$, the ΣCFTE29o$^-$ cell line continues to express increasing levels of HO-1. Prolonged expression of HO-1 in response to transient oxidative stress may provide some explanation for elevated CO observed in CF compared to normal controls (Horvarth *et al.,* 1998).

In summary, this study demonstrates that the anti-oxidant enzyme HO-1 is expressed by human tracheal epithelial cell lines ΣCFTE29o$^-$ and 9HTEo$^-$ under basal conditions and cannot be up-regulated by pro-inflammatory cytokines. While 15d-$PGJ_2$ can be observed to induce an increase in HO-1 expression, the high concentrations of this PG needed to elicit a response are unlikely to be of physiological relevance. Interestingly while differences in the basal expression of HO-1 expressed by ΣCFTE29o$^-$ and 9HTEo$^-$ cells cannot offer any explanation as to the source of increased CO levels observed in the exhaled breath of CF patients compared to non-CF individuals (Horvarth *et al.*, 1998), a prolonged pattern of expression of HO-1 by the CF airway epithelium compared to the non-CF airway epithelium may provide a mechanism behind this phenomenon.

## 6.3 Summary of Results

- HO-1 is protein is constitutively expressed, at similar levels, in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines
- Pro-inflammatory cytokine TNFα does not appear to induce HO-1 protein in 9HTEo$^-$ or ΣCFTE29o$^-$ cells over 24 hours
- $PGE_2$, in micro-molar concentrations, does not affect HO-1 protein expression at a 24 hour time point in either cell line
- At a 24 hour time point 1 and 10 μM 15d-$PGJ_2$ induce HO-1 protein expression by the ΣCFTE29o$^-$ cell line. No increase in HO-1 expression is observed at this time point in response to 15d-$PGJ_2$ in 9HTEo$^-$ cells
- HO-1 protein expression is induced , compared to basal, by stimulation with 10μM 15d-$PGJ_2$ in 9HTEo$^-$ cells at a 6 hour time point but not at 2, 14, and 24 hour time points
- In ΣCFTE29o$^-$ cells HO-1 protein expression is elevated, compared to basal, in cells stimulated for 14 and 24 hours with 10 μM 15d-$PGJ_2$
- Troglitazone (10 μM) has no significant effect on HO-1 expression in either cell a 2, 6, 14 or 24 hour time points
- $PGE_2$ (10 μM) does not significantly affect the basal metabolic activity of either the HTEo$^-$ or ΣCFTE29o$^-$ cell lines
- Troglitazone (10 μM) does not significantly affect the basal metabolic activity of either the 9HTEo$^-$ or ΣCFTE29o$^-$ cell lines
- 15d-$PGJ_2$ (10 μM) significantly reduces the basal metabolic activity of the 9HTEo$^-$ cell line at the 6 hour time point
- 15d-$PGJ_2$ (10 μM) does not significantly affect the basal metabolic activity of ΣCFTE29o$^-$ cells at any of the time points observed in this study

# 7. General Discussion

In CF, defective function of the CFTR in airway epithelial cells and submucosal glands results in chronic disease of the airways leading to deterioration of lung function and ultimately death (Van Heeckeren, 1997). An imbalance in the ionic and/or osmotic balance of the ASL results in clogging of the airways with thick, sticky mucus, and facilitates colonisation by bacteria such as *Burkholderia cepacia, Staphylococcus aureus,* and *Psudomonas aeruginosa.* The CF airway mounts a vigorous inflammatory response to the bacteria, which in early life is often successful in preventing chronic colonisation (Dakin *et al.,* 2002). However, eventually the CF lung becomes unable to clear infection and bacterial colonisation of the airway becomes chronic. Konstan *et al.* (1994) report that older children and adults with CF have large numbers of bacteria in their lower airways accompanied by neutrophil influx and uninhibited elastase activity. Furthermore, a number of studies report that a modest inflammatory response can be observed in the airways of CF infants in the absence of a bacterial infection (Reviewed by Chmiel and Davies, 2003), this suggests that inflammation may be intrinsic in CF airways or that the inflammatory response mounted by the CF airway in response to bacterial infection is not down-regulated once the pathogen has been eliminated. A persistent and exaggerated inflammatory response, associated with elevated levels of cytokines, is one of the major factors leading to damage of the host tissue in CF (Escotte, 2002). DiMango *et al.* (1995) report that CF cell lines produce higher levels of IL-8, in response to *Pseudomonas* stimulation, than non-CF cells. Additionally, Thomas *et al.* (2000) demonstrate that CF mice show increased production of TNFα, in response to challenge with bacterial LPS, than their non-CF littermates. Furthermore, a number of studies have reported elevated levels of PGs in the sputum of CF patients when compared to healthy controls (Zakrzewski *et al.*, 1987; Strandvik *et al.,* 1996). Ultimately, the chronic and excessive inflammatory response seen in CF airways leads to destruction of host tissue and pulmonary failure. Consequently, the host inflammatory response is viewed as a therapeutic target with potential to delay the deterioration in lung function seen in CF.

Elevated levels of PGs observed in the sputum of CF patients compared to healthy controls suggest a role for PGs in the excessive inflammatory response, therefore

elucidation of the role and regulation of PG production by airway epithelial cells may provide insight into more effective anti-inflammatory therapies for diseases of the airways such as CF. In light of these findings, this study set out to investigate the role of tracheal epithelial cell lines in the production of PGs, the expression and regulation of enzymes involved in PG production, and to define which agents might affect their expression.

$PGE_2$ is the most common PG in the airways and is believed to be predominantly a COX-2 product. The airway epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$ produced $PGE_2$ constitutively. Interestingly, the ΣCFTE29o$^-$ produced significantly higher levels of $PGE_2$ than the non-CF phenotype 9HTEo$^-$ cell line. This data suggests that the airway epithelium could be the souce of elevated PG levels seen in CF and supports the clinical findings of Zakrzewski *et al.* (1987) and Strandvik *et al.* (1996) who report elevated levels of PGs in the sputum of CF patients compared to healthy controls, and the work of Freedman *et al.* (1999 and 2002) who report an elevated level of phospho-lipid bound AA and an increase in levels of $PGE_2$ in the BAL fluid from *cftr*$^{-/-}$ mice when compared to wild-type controls. This was not the case, however, for IL-8. No significant difference in basal IL-8 production could be observed between the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. While some differences could be observed between IL-8 production in these cell lines in response to stimulation with pro-inflammatory cytokines, the disparity was not highly significant nor did one cell line demonstrate consistently higher levels than the other, suggesting that differences in IL-8 production observed in response to cytokines may be a result of differences in cytokine receptor expression rather than IL-8 being more inducible in one cell line over the other. The ability of pro-inflammatory cytokines to significantly up-regulate IL-8 production by the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines confirms that the airway epithelium can be a source of IL-8 in the setting of airway inflammation and that the cytokine signalling and NFκB activation pathways must be intact in these cells. These data are in line with the findings of Becker *et al.* (2003) who report that primary human airway epithelial cell lines with a CF- and non-CF phenotype generally display similar patterns of IL-8 production and NFκB activation at baseline or in response to a diverse set of relevant stimuli. The study by Becker *et al.* does, however, report that IL-8 production by the

CF cell line is exaggerated and sustained in response to *P. aeruginosa* in the presence but not in the absence of serum. These data suggest that baseline differences in inflammation due to mutant CFTR are not a primary cause for the hyperinflammatory status of the CF lung. But support the hypothesis that severe inflammation occurs in response to bacteria, their products and that CF cells are less capable of downregulating proinflammatory responses.

In line with the findings of Mitchell *et al.,* 1994; Asano *et al.,* 1996; Watkins *et al.,* 1999; and Rodgers *et al.,* 2002, this study found that the airway epithelial cells observed constitutively expressed both COX-1 and COX-2. Interestingly, while no difference in COX-2 expression was observed between the CF and non-CF phenotype cell lines, 9HTEo$^-$ cells constitutively expressed higher levels of COX-1 protein than ΣCFTE29o$^-$ cells, despite ΣCFTE29o$^-$ cells producing approximately eight times more $PGE_2$ than 9HTEo$^-$ cells. In contrast to the findings of the aforementioned studies, COX-2 protein expression could not be up-regulated by stimulation of the cell lines with pro-inflammatory cytokines. The constitutive expression of COX-2 and rapid up-regulation of $PGE_2$ by human tracheal epithelial cells suggests that $PGE_2$ expression by the tracheal epithelium may be part of a natural defense mechanism as well as a contributor to airway inflammation, as implied by elevated levels of this PG detected in the CF Phenotype cell line when compared to the non-CF cell line. The potential for COX-2 to be playing a homeostatic role in the airways suggests that, under certain circumstances, its inhibition may be deleterious. Reuter *et al.* 1996 report that in animal models of pre-existing gastrointestinal injury and inflammation daily treatment with a selective inhibitor of COX-2 (L745337) at doses that did not inhibit COX-1 resulted in significant inhibition of mucosal prostaglandin synthesis and a marked increase in the severity of colonic damage. These findings may also of relevance to the CF airway where a persistent inflammatory response, featuring elevated levels of PGs, leads to tissue damage but COX-2 expression is constitutive and may play a homeostatic role. It would, therefore, be of interest to assess the affect of COX-2 inhibition in subjects with chronic inflammation of the airways.

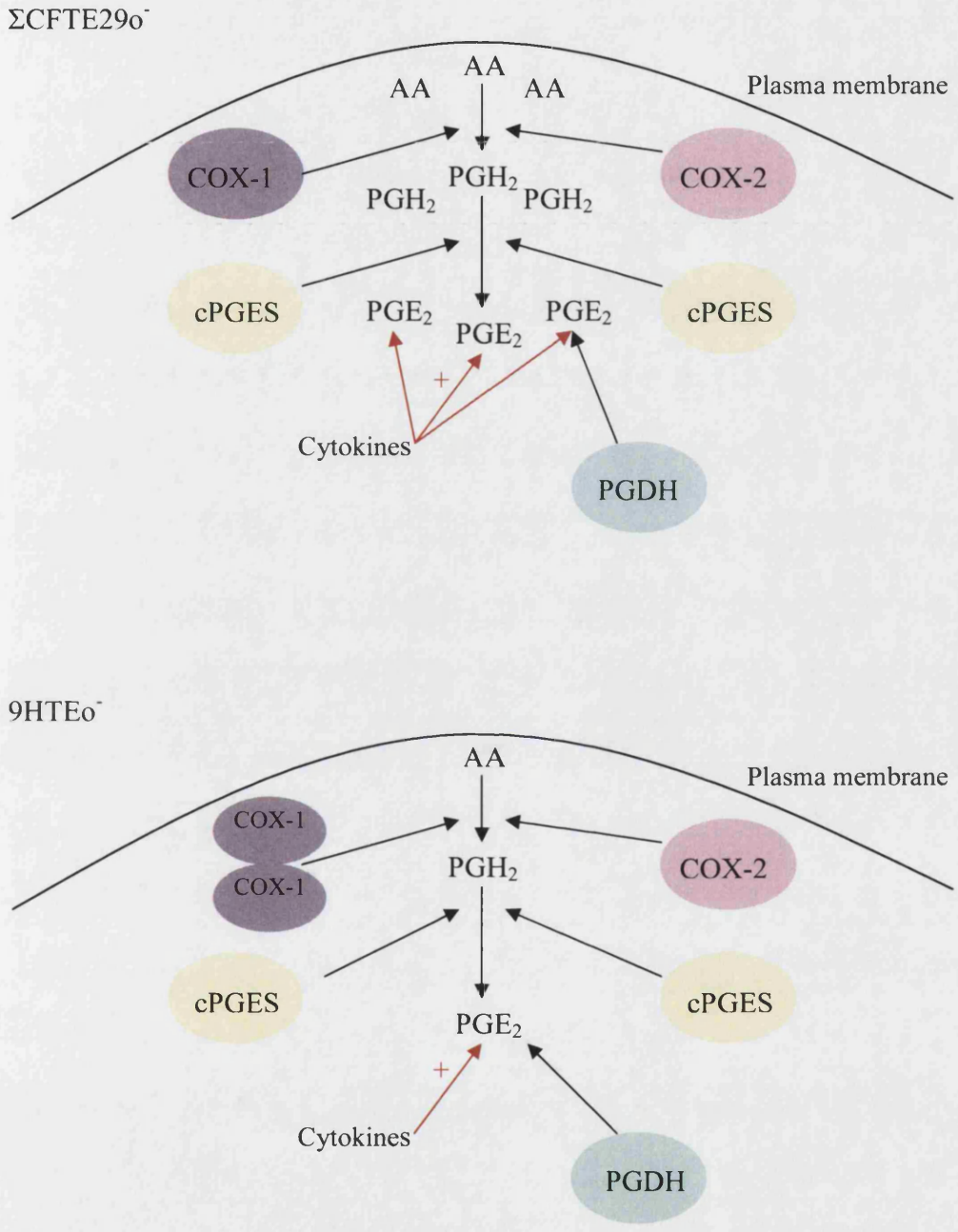
The role of PGs in the airway is complex and their effects may be pro- or anti-inflammatory dependent upon the time point during inflammation, the receptor sub-

types activated and the model being observed. The up-regulation of $PGE_2$, by the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, at the 2 hour and 6 hour time points after stimulation with pro-inflammatory cytokines suggests a pro-inflammatory role for this PG in the human tracheal epithelium. This finding raises the possibility that the specific PGE synthases may be a valid therapeutic target in airway inflammation. As previously mentioned, three major isoforms of PGES have been identified to date cPGES a reportedly constitutive isoform functionally coupled to COX-1 (Murakami *et al.,* 2000), an inducible isoform, mPGES, coupled to COX-2 (Murkami *et al.,* 2000), and mPGES-2 a constitutive isoform that promotes $PGE_2$ production via both COX-1 and – 2 (Murakami *et al.,* 2003). The apparent absence of mPGES in human tracheal epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$, indicates that COX-2 may be coupling with cPGES or mPGES-2 to produce $PGE_2$. The ability of cPGES to functionally pair with COX-2 is supported by the findings of Han and Smith (2002) who report the absence of mPGES in KAT-50 thyrocytes and postulate that in its absence, cPGES and COX-2 form an inefficient couple resulting in low $PGE_2$ production despite high expression of COX-2. It was not possible to determine the expression of mPGES-2 protein at the time this study was conducted, as the relevant anti-body was not available.

In line with the finding that $PGE_2$ is constitutively expressed by both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines was the observation that the $PGE_2$ metabolising enzyme PGDH is constitutively expressed by these cells. No difference in the expression or regulation of PGDH could be detected between the tracheal epithelial cell lines used in this study. This indicates that the higher levels of $PGE_2$ produced by the ΣCFTE29o$^-$ cell line when compared to the 9HTEo$^-$ cell line are not a consequence of elevated PGDH expression in 9HTEo$^-$ cells. This study did not investigate the activity of PGDH and it is possible that differences in the activity of PGDH between the cell lines may, in part, contribute to the disparity in $PGE_2$ production observed. However, the ability of pro-inflammatory cytokines TNFα and IL-1β to up-regulate $PGE_2$ production by both 9HTEo$^-$ and ΣCFTE29o$^-$ cells despite having no effect upon the expression of COX, PGES, or PGDH protein expression suggests that they are modulating the release of substrate from the cell membrane rather than affecting enzymes downstream of AA. These findings are summarised in figure 68. Pro-inflammatory cytokines IFNγ, TNFα, and IL-1β are reported to up-regulate/activate PLAs in airway epithelial cells (Wu *et al.,*

1997; Yao *et al.,* 1998). It is therefore possible that the increases in $PGE_2$ production, induced by pro-inflammatory cytokines, in the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines is a result of elevated PLA expression and/or activity. However, the pattern of induction of $PGE_2$ and the fold-increase from basal of $PGE_2$ production, in response to pro-inflammatory cytokines, is similar for both cell lines. These data suggest that the difference in $PGE_2$ production is likely to be a consequence of increased AA availability in the CF Phenotype ΣCFTE29o$^-$ cell line compared to the non-CF phenotype 9HTEo$^-$ cell line. This hypothesis is supported by the findings of Freedman *et al.,* (1999; 2002) who, as previously stated, report an elevated level of phospho-lipid bound AA and an increase in levels of $PGE_2$ in the BAL fluid from *cftr*$^{-/-}$mice when compared to wild-type controls. In their 2002 study Freedman *et al.* report that oral administration of DHA resulted in a selective decrease in eicosanoid formation in *cftr*$^{-/-}$ mice that was not observed in WT mice suggesting that the membrane lipid imbalance observed in lungs from *cftr*$^{-/-}$ mice may play an important role in the pathogenesis of the enhanced pulmonary inflammation observed in CF. The findings in this study add to the body of evidence that $PGE_2$ levels are elevated and contribute to the inflammatory phenotype seen in CF. Furthermore, these data provide evidence to suggest that the disparity observed in PG levels between cells of a CF and non-CF phenotype are a result of factors upstream of the COX enzymes in the PG biosynthetic pathway.

Figure 68 A model of $PGE_2$ formation by human tracheal epithelial cell lines.




Abbreviations: Arachidonic acid, AA; cyclooxygenase-1, COX-1; cyclooxygenase-2, COX-2; cytosolic prostaglandin E synthase, cPGES; 15-hydroxyprostaglandin dehydrogenase, PGDH; Prostaglandin $E_2$, $PGE_2$; Prostaglandin $H_2$

Konstan *et al.* (1995) report that in patients with cystic fibrosis and mild lung disease, high-dose ibuprofen, taken consistently for four years, significantly slows the progression of the lung disease. However, despite the ability of high dose ibuprofen to retard progression of lung disease, adverse events, such as renal toxicity (Scott *et al.,* 2001) and potentiation of intestinal damage caused by pancreatic enzyme treatment (Kimura *et al.,* 1999), mar its therapeutic utility. Therefore, the development of alternative anti-inflammatory agents is necessary. PPARs are ligand activated transcription factors belonging to the nuclear receptor superfamily. A growing body of evidence suggests that ligands of PPARγ may be suitable anti-inflammatory agents and provide an alternative to the currently used therapies for inflammation control. A number of studies have reported that both natural and synthetic ligands for PPARγ possess anti-inflammatory properties, attenuating the release of cytokines, NOS and COX-2 in a number of cell types (Jiang *et al.,* 1998; Ricote *et al.,* 1998; Welch *et al;* 2003; Colville-Nash *et al.,* 1998; Patel *et al.,* 2003). Wang *et al.* (2001) report that activation of PPARγ in airway epithelial cells dramatically inhibits cytokine-induced expression of inflammatory mediators by these cells and suggest that PPARγ may act as a negative immunomodulator in the airways. Patel and co-workers (2003) demonstrate that PPARγ ligands suppress cell growth, induce apoptosis and inhibit GM-CSF release from HASM cells, supporting the hypothesis that PPARγ may be a potential target for therapies aimed at inflammatory disorders of the airways. However, the fact that PPARγ ligands are also able to up-regulate COX-2 expression on colonic epithelial cell and mammary epithelial cells (Meade *et al.,* 1999) suggests that the effects of PPARγ in inflammation are tissue specific and require elucidation.

In line with the finding that airway epithelial cells express PPARγ (Michael *et al.,* 1997; Wang *et al.,* 2001) this study found that PPARγ was expressed by both 9HTEo$^-$ and ΣCFTE29o$^-$ cells. Interestingly, the CF Phenotype cell line expressed significantly higher levels of this receptor than the non-CF phenotype tracheal epithelial cell line. As the cell lines used in this study are not a genetically matched pair, it is difficult to draw any conclusions as to the relevance of the high expression in the CF Phenotype cell line. 15d-$PGJ_2$, a degradation product of $PGD_2$, is an endogenous high affinity ligand for PPARγ (Forman *et al.,* 1995; Kliewer *et al.,* 1995). The low levels of $PGD_2$ observed to be produced by the cell lines used in this study suggest that very little 15d-$PGJ_2$

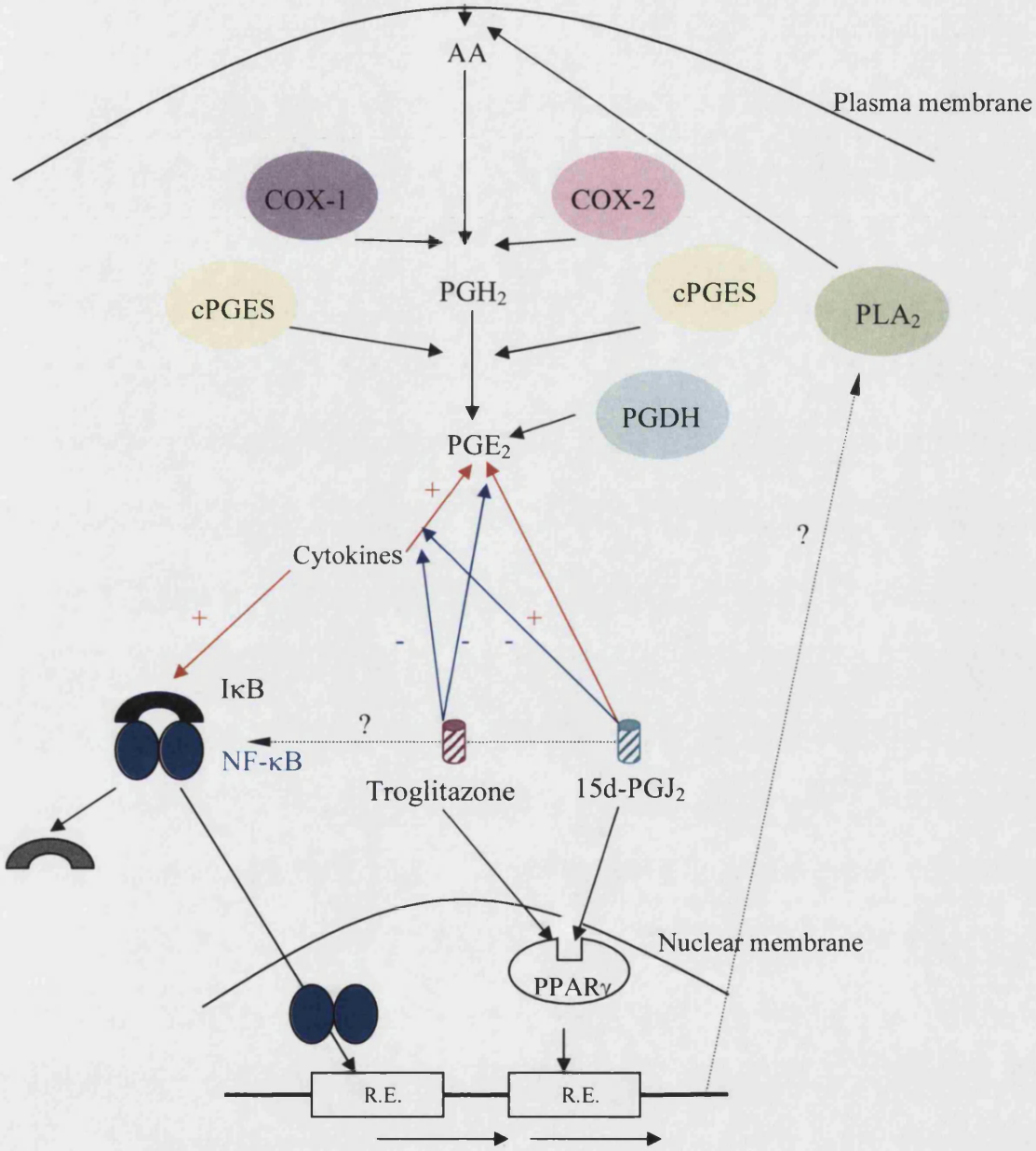
would be present in the cells' milieu and therefore in these cell lines, it would not be an endogenous activator of PPARγ. In this study, exogenously applied 15d-$PGJ_2$, troglitazone, and PPARγ antagonist BADGE, at concentrations ranging from pM to μM, could not be observed to have any effect on expression of the enzymes in the $PGE_2$ biosynthetic pathway at protein level. However, 15d-$PGJ_2$ (10μM) induced an increase in basal $PGE_2$ production by both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Conversely, troglitazone appeared to inhibit $PGE_2$ generation by both cell lines. This may indicate that the effect of 15d-$PGJ_2$ on $PGE_2$ generation is PPARγ-independent. Castrillo *et al.*(2000) and Chawla *et al.*(2001) demonstrate that 15d-$PGJ_2$ is able to elicit responses in cells that do not express PPARγ and state that its role in inflammation is attributable not only to its binding and activating PPARγ but to its ability to modulate the activity of the transcription factors including NFκB. This group also state that *in vitro* concentrations of 15d-$PGJ_2$ of 2.5μM and above are required to ligate the proteins in the NFκB signalling pathway. Therefore, it is possible that the concentration of 15d-$PGJ_2$ used in this study could be inducing $PGE_2$ by modulating the NFκB pathway. However, IL-8 transcription is regulated by NFκB, and at a concentration of 10μM, 15d-$PGJ_2$ had no effect on basal IL-8 generation by either cell line used in this study suggesting that the basal activity of NFκB is not being affected by this ligand at the concentrations used in this study. These findings are summarised in figure 69.

The ability of 15d-$PGJ_2$ to induce basal $PGE_2$ production but to inhibit TNFα induced $PGE_2$ generation is of interest as it suggests that this ligand may regulate $PGE_2$ generation via a negative feedback-loop. The control of COX-2 expression by a negative-feedback loop mediated through PPARγ has been demonstrated by Inoue *et al.* (2000) in the macrophage-like cell line U937. As previously stated, in the cell lines used in this study, 15d-$PGJ_2$ was not observed to have any effect upon COX-2 protein expression. However, this study did not look at the expression of the PLA enzymes. Alaoui-El-Azher *et al.* (2002) report that 15d-$PGJ_2$ mediates inhibition of LPS-stimulated sPLA(2)-IIA expression via a PPAR-gamma-dependent pathway. It is therefore possible that 15d-$PGJ_2$ is affecting $PGE_2$ generation by modulating expression of PLA enzymes.

The ability of 15d-$PGJ_2$ to both up- and down-regulate $PGE_2$ production by human tracheal epithelial cell lines requires further investigation. As the CF airways have been demonstrated to produce elevated levels of PGs (Zakrzewski *et al.*, 1987; Strandvik *et al.,* 1996) and these PGs may be contributing to the excessive inflammatory response. The use of pharmacological use of PPARγ ligands as anti-inflammatory therapy in the CF airway, could in some circumstances be detrimental to health. Elucidation of the mechanisms by which PPARγ ligands regulate $PGE_2$ production in these cell lines may clarify the suitability of these ligands to be used in the inflammatory setting of CF.

The data gathered from the 9HTEo$^-$(1) and ΣCFTE29o$^-$(2) cell lines, with respect to the effect of PPARγ ligands on COX-2 expression, differs markedly from the data obtained from the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. After problems with the growth of the 9HTEo$^-$(1) and ΣCFTE29o$^-$(2) cells a new batch of each cell line, 9HTEo$^-$ and ΣCFTE29o$^-$, was procured from the original source (generously supplied by D.C. Gruenert, University of San Francisco, CA). The growth pattern and data obtained from the new cells was consistent. Additionally, the ability of pro-inflammatory cytokines to up-regulate $PGE_2$ production, absent in 9HTEo$^-$(1) cells, suggests that the PG biosynthetic pathway is intact in these cells. Therefore, the data obtained from the old batch of cells was disregarded. The difference in data and problems with the growth suggest that caution should be employed when using these cell lines to investigate the effect of PPARs on PG generation as age related changes to the cell lines appear to affect these pathways.

Figure 69 Model of effect of PPARγ ligands on the $PGE_2$ biosynthetic pathway in 9HTEo$^-$ and ΣCFTE29o$^-$ cells




Abbreviations: Arachidonic acid, AA; cyclooxygenase-1, COX-1; cyclooxygenase-2, COX-2; cytosolic prostaglandin E synthase, cPGES; inhibitory kappa B, IκB; 15-hydroxyprostaglandin dehydrogenase, nuclear factor kappa B, NFκB; PGDH; Peroxisome proliferator activated receptor gamma, PPARγ; Prostaglandin $E_2$, $PGE_2$; Prostaglandin $H_2$; Phospholipase $A_2$, $PLA_2$; response element, R.E.

HO-1 is an inducible anti-oxidant enzyme with anti-inflammatory properties mediated by the ability of its enzymatic products to down-regulate the inflammatory response (Poss *et al.,* 1997; Li Volti *et al.,* 2003). A study by Horvarth *et al.* (1998) reports elevated levels of exhaled CO in the breath of CF patients which may be of pathophysiological significance in patients with this disease. Horvarth *et al.* postulate that HO-1 is the source of this CO. Furthermore, Donnelly and Barnes (2001) put forward the hypothesis that the airway epithelium may be the source of increased CO in airway diseases. The aforementioned report finds that primary human airway epithelial cell lines express low levels of HO-1 constitutively and that the expression of this enzyme can be increased upon incubation of the cells with a mixture of pro-inflammatory cytokines. The increase in HO-1 protein expression was observed at the 4 hour time point and had decreased by the 12 hour time point. In line with this report, this study finds that airway epithelial cell lines 9HTEo$^-$ and ΣCFTE29o$^-$ express similar levels of HO-1 under basal conditions. However, in contrast to the findings of Donnelly and Barnes, the expression of this enzyme could not be induce by incubation of the cells with pro-inflammatory cytokines, either alone or in combination. It is possible that the constitutive expression of HO-1 by human tracheal epithelial cell lines is a consequence of continual low level of cytokine production by these cells.

Zhou *et al.* (2004) state that that the induction of HO-1 in patients with CF is a cytoprotective event and that augmenting its expression may be a potential therapy against bacterial injury. A number of studies have illustrated that 15d-$PGJ_2$ induces HO-1 expression in a variety of cell systems (Colville-Nash *et al.,*1998; Koppal *et al.,* 2000; Wayman *et al.,* 2002; Lee *et al.,*2003; Alvarez-Maqueda *et al.,* 2004). This study demonstrates that micro-molar concentrations of 15d-$PGJ_2$ induce HO-1 expression by both 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, it should be noted, however, that concentrations of 15d-$PGJ_2$ in the micro-molar range are more likely to be of pharmacological than physiological relevance. The finding that HO-1 expression was induced by 15d-$PGJ_2$ suggests that 15d-$PGJ_2$ may be inducing intracellular oxidative stress. HO-1 was transiently increased in the non-CF phenotype cell line, increasing at the 6 hour time point and returning to basal levels by the 14 hour time point. Concomitantly, the basal metabolic activity of this cell line dropped at the 6 hour time point but returned to levels not significantly different from normal by the 14 hour time point. Interestingly,

induction of HO-1 in the CF Phenotype cell line occurred at a later time point after incubation with 15d-$PGJ_2$ than in the non-CF phenotype cell line. Furthermore, HO-1 expression by the ΣCFTE29o$^-$ cell line continued to increase at the 24 hour time point. However, the metabolic activity of these cells did not appear to be affected by this ligand. These data suggest that 15d-$PGJ_2$ induces oxidative stress resulting in increased HO-1 expression in human tracheal epithelial cell lines, but that while this event is transient in the non-CF phenotype cell line the effects are sustain in the CF Phenotype cells. As stated by Horvarth *et al.* (1998) HO-1 may be a source of increased CO in the exhaled breath of CF patients when compared to healthy controls and as such may be contributing to the pathophysiology of this disease. This study demonstrates that whilst differences in basal expression of HO-1 between the CF Phenotype airway epithelial cell line and the non-CF Phenotype airway epithelial cell line do not offer any explanation for the increased levels of CO observed in exhalations from CF patients, the prolonged induction of HO-1 CF in airway epithelial cells, compared to non-CF airway epithelial cells, in response to transient oxidative stress may provide insight into a mechanism behind this phenomenon. These data also support the hypothesis of Donnelly and Barnes (2001) who postulate that the airway epithelium is the source of elevated CO in CF airways.

Once again, these findings raise questions about the relevance of PPARγ ligands as anti-inflammatory agents in the CF lung. In the non-CF cell line, 9HTEo$^-$, these ligands appear to have anti-inflammatory activity, decreasing both basal and pro-inflammatory cytokine induced $PGE_2$ generation and transiently up-regulating expression of HO-1, which may prove beneficial in an inflammatory setting. However, in the CF Phenotype cell line, ΣCFTE29o$^-$, the contradictory effects of PPARγ ligands 15d-$PGJ_2$ and troglitazone on basal $PGE_2$ generation and the sustained induction of HO-1 by 15d-$PGJ_2$ suggest that these ligands may exacerbate the existing inflammatory condition and cause further damage to the host.

# 8. Conclusions

This study demonstrates that, in line with the finding that PG levels in the sputum from CF patients are elevated when compared to healthy controls, the CF phenotype human tracheal epithelial cell line ΣCFTE29o$^-$ produces significantly more $PGE_2$ under basal conditions than the non-CF phenotype cell line 9HTEo$^-$. These findings provide evidence that the airway epithelium is potentially a major source of $PGE_2$ in the airways and implicate the airway epithelium as a contributor to the excessive inflammatory response observed in the CF lung.

Despite the fact that the ΣCFTE29o$^-$ cell line produces significantly higher levels of $PGE_2$ than the 9HTEo$^-$ cell line, no significant difference in the expression of $PGE_2$ biosynthetic enzymes could be observed between the cell lines to explain this disparity. The airway epithelial cells used in this study were found to constitutively express both COX-1 and COX-2 proteins. The PGES cPGES was constitutively expressed by both cell lines. Interestingly, the COX-2 coupled mPGES was absent from both cell lines and its expression could not be induced by any of the ligands used in this study. Therefore, the production of $PGE_2$ by the human tracheal epithelial cell lines used in this study may be a product of the action of COX-2/cPGES coupling or the functional coupling of COX-2 /mPGES-2. At the time this study was conducted, there was not an antibody for mPGES-2 available to investigate this further. It would be of interest to investigate the expression and regulation of this enzyme in these cell lines. As the time scale of $PGE_2$ induction by these cell lines suggest that it is acting as a pro-inflammatory mediator, elucidation of the role and regulation of the PGES enzymes may provide insight into therapeutic targets aimed at reducing pro-inflammatory PG production whilst maintaining levels of homeostatic PGs. Expression of the $PGE_2$ metabolising enzyme PGDH was constitutive in both cell lines and also could not be induced by pro-inflammatory stimuli. This finding suggests that elevated expression of his enzyme cannot be implicated as a contributory factor to the difference in $PGE_2$ expression observed between the ΣCFTE29o$^-$ and 9HTEo$^-$ cell lines.

The finding that enzymes of the $PGE_2$ biosynthetic pathway are expressed at similar levels by both cell lines and that, despite differences in the level of $PGE_2$ production by

these cell lines, a similar fold increase in $PGE_2$ generation can be observed in response to pro-inflammatory cytokines, suggests that the high levels of $PGE_2$ generated by the CF phenotype cell line are a result of elevated AA rather than the expression or activity of down-stream enzymes.

Human airway epithelial cell lines were found to constitutively express the nuclear hormone receptor transcription factor PPARγ. The ΣCFTE29o$^-$ cell line expressed higher levels of this receptor than the 9HTEo$^-$ cell line, whether this finding has any significance with regards to CF cannot be concluded from these data as the cell lines used for this study are not a genetically match pair. Despite the ability of PPARγ ligands to inhibit $PGE_2$ generation and transiently increase HO-1 expression by the non-CF phenotype cell line, the conflicting effects of PPARγ ligands on the production of $PGE_2$ and the induction of sustained HO-1 expression in CF phenotype cells, indicate that use of these ligands may not be suitable anti-inflammatory agents in the CF lung and may exacerbate the exaggerated inflammatory response already present. Further investigation is required into the exact mechanisms behind the action of PPARγ ligands on PG production in airway epithelial cells, particularly in an inflammatory setting.

# 9. Appendices

## 9.1 Buffers and Solutions

### 9.1.1 Solutions for tissue culture

Tissue culture reagents were used for all cell cultures and solutions were prepared using sterile MilliQ water.

| **Phosphate Buffered Saline, pH 7.4** | **CFTE and HTE medium - MEM** |
|---|---|
| 140 mM NaCl<br>2.7 mM KCl<br>1.5 mM $KH_2HPO_4$<br>8.1 mM $Na_2HPO_4$ | Supplemented with penicillin (10 units/ml), streptomycin (10 units/ml) and fungizone (0.5 µg/ml) |
| **Trypsin – EDTA Solution** | **Freeze medium** |
| 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA | 10% (v/v) DMSO<br>40% (v/v) FBS<br>50% (v/v) CFTE/HTE medium |

### 9.1.2 Solutions for SDS-PAGE and Western blotting

| **Lysis Buffer** | **Stock solutions** |
|---|---|
| 50 mM Tris-HCl pH 7.5 (stock 1 M) | 1 M Tris-HCl pH 8.8 |
| 150 mM NaCl (stock 5 M) | 1 M Tris-HCl pH 6.8 |
| 1% (v/v) Nonidet P40 (stock 10%(v/v)) | 10% (w/v) SDS |
| 10% glycerol | 10% (w/v) ammonium persulfate |
| 5 mM EDTA (stock 0.5 M, pH 8.0) | TEMED |
| 1 mM sodium orthovanadate | |
| 1 mM sodium molybdate | |
| 10 mM sodium fluoride | |
| 40 µg/ml phenylmethylsulfonyl fluoride (PMSF) | |
| 1 µg/ml pepstatin A | |
| 10 µg/ml aprotinin | |
| 10 µg/ml leupeptin | |
| 10 µg/ml soyabean trypsin inhibitor | |
| MilliQ water | |

| **SDS-PAGE running buffer** | **5X SDS-sample buffer** |
|---|---|
| 25 mM Trizma base | 5% SDS |
| 192 mM glycine | 50% glycerol |
| 0.1% (w/v) SDS | 200 mM Tris-HCl pH 6.8 |
| MilliQ water | MilliQ water |
| | 5% 2-mercaptoethanol |
| | Bromophenol blue |
| | Store in dark |
| **Wet transfer buffer** | **Tris-buffered saline (TBS)** |
| 39 mM glycine | 20 mM Tris-HCl pH 7.5 |
| 48 mM Trizma base | 150 mM NaCl |
| 20% (v/v) methanol | MilliQ water pH 7.5 |
| **Ponceau S solution** | **Blocking buffer** |
| 0.1% (w/v) Ponceau S | 5% (w/v) non |
| 5% (v/v) Acetic acid | -fat powdered milk (Marvel) in TBS |
| **7.5% w/v Acrylamide running gel (10ml)** | **5% w/v Acrylamide stacking gel (8ml)** |
| 3.75ml Protogel {30%(w/v) acrylamide, | 1.33ml Protogel {30%(w/v) acrylamide, |
| 0.8% (w/v) bisacrylamide | 0.8% (w/v) bisacrylamide |
| 1M TRIS-HCl, pH 8.8 | 1M TRIS-HCl, pH 6.8 |
| 0.1% SDS | 0.1% SDS |
| 0.1% ammonium persulphate | 0.1% ammonium persulphate |
| 5.6ml Milli-Q water | 6.36ml Milli-Q water |
| 20 µl N,N,N',N', tetramethylene-diamine | 20 µl N,N,N',N', tetramethylene-diamine |
| (TEMED) | (TEMED) |

## 9.2 Antibodies used for immunoblotting

| Antigen | Molecular weight (kDa) of antigen | Secondary antibody |
|---|---|---|
| COX – 1 | 68 – 74 | Anti-goat HRP |
| COX – 2 | 68 – 74 | Anti-goat HRP |
| Pan ERK 1/2 | 42 / 44 | Anti-rabbit HRP |
| PPARγ | 50 – 54 | Anti-rabbit HRP |
| cPGES | 23 | Anti-rabbit HRP |
| mPGES | 16 | Anti-rabbit HRP |
| PGDH | 29 | Anti-rabbit HRP |
| HO-1 | 32 | Anti-rabbit HRP |

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